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OCTOBER, 1951

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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

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Endowment fund of the International Association for Dental Research (as of June 1, 1949): $1,506.06. There have been no expenditures from this fund.

Notices

The 30th General Meeting of the International Association for Dental Research will be held at the Broadmoor, Colorado Springs, March 21, 22, and 23, 1952.

Subsection Nd-Dentistry—of the A.A.A.S. will meet in Philadelphia on December 28 and 29. A symposium on Fluoridation as a Public Health Measure will be presented.

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THE EDITOR'S VIEWPOINT

Linghorne and O'Connell (p. 604), from a study of experimentally produced periodontal defects in dogs, found the presence of calcified tissue to be a factor in differentiation of osteoblasts. They suggest that resorbing calcified tissues may supply the stimulus. They observed regeneration of alveolar bone. To the periodontist who hopes to find repair under ideal conditions this is an important finding.

Many impressions have been passed on from one generation of practitioners to the next without scientific evaluation. Quite often careful experiment confirms the clinical impressions and empiric teachings. The uninitiated may look on such investigation as a waste of time, money, and energy. Often enough, to justify such studies of "accepted fact," the results are similar to those reported by Ramfjord (p. 615). A study of the gingiva and periodontium by observation, x-ray, and microscopy showed no changes attributable to an acute febrile disease (poliomyelitis) in monkeys. Ramfjord suggests that the clinical impression that acute disease causes periodontal disease may be due to the accumulation of debris on the teeth and gingivae, associated with neglect of oral hygiene, during acute illness. The report that certain phases of traumatic lesions observed resemble lesions described as periodontosis is worthy of further consideration.

Cattoni (p. 627) presents evidence of lymphocytes in the epithelium of healthy gingivae and discusses their possible defense role. It is worth remembering that inflammation is not a pathologic process but is the response of normal tissues to irritation. Since the typical cells of the inflammatory process are essential for defense it should be no more amazing to find them in small numbers than to find a small standing army in a nation at peace but prepared for self-defense.

Zwirner (p. 638) describes an apparatus for examination of the movement of rat's teeth in their alveoli. The dynamics of the dento-alveolar joint have been less enthusiastically investigated than its micromorphology. Studies of the minute movements of the teeth should be useful in partial dental prosthesis, crown and bridge prosthesis, and orthodontics.

Bartels (p. 642) describes the formation of calcium carbonate crystals by bacteria which normally inhibit the mouth and the dentobacterial plaque. In vitro, they produced crystals on suitable media. Bartels believes that this is collaborative evidence of Naeslund's theory of calculus as a metabolic product of bacteria. Certainly, bacteria are essential to calculus production; their metabolic activity may in some instances lead to calculus formation and in others to acid production.

Davies and King (p. 645) studied the effect of ammonium ion dentifrice on the control of dental caries under usual conditions of use. We have long been impressed with the artificial conditions under which dentifrices have been tested recently, i.e., at specified times under supervision. It has seemed to us that while these studies might show the relative effectiveness of dentifrice *X* against a control under the special conditions, they might not pertain when the dentifrice *X* is sold across the drugstore counter as a result of television, radio, and newspaper advertising plus the popular pseudo-science contained in periodicals designed for lay consumption. Davies and King found ammonium ion dentifrice ineffective when used routinely over a one-year period. Their well prepared report is worthy of careful study.

Pearlman (p. 656), continuing the study of the influence of ammonia and lactobacilli, implies that the introduction of ammonium phosphate and urea into the mouth should not be expected to cause immediate inhibition of acid production unless high concentrates are maintained and the reaction is kept alkaline. The results do not appear too encouraging for advocates of these substances in caries' control.

Scrivener, Myers, Moore, and Warner (p. 665) show evidence of antagonism and satellitism between the oral flora and lactobacilli. These investigators have interested themselves in this problem for sometime. The complexity of the oral flora makes definitive investigation of the problem difficult but the possible significance is undeniable.

In a report on caries activity in 12-, 13-, and 14-year-old children after 23 and 34 months of water fluoridation, Hill, Blayney,

and Wolf (670) show 12 per cent reduction in D.M.F. They, again, question how much of this reduction can be attributed to fluoridation of water alone. It seems most amazing that caries immunes among 13-year olds increased by 146 per cent but when it is realized that this is only a change from 1.02 immune children per hundred to 2.51 immunes per hundred the figure becomes less impressive. This factual report is worthy of close study.

Martin (p. 676) found that fresh and frozen vegetables cooked in fluoride-containing waters absorb fluorine. These studies are adding to our knowledge of the fluoridation problem. Protests have been made by at least one religious group. Suggestions, based on nothing but guesses, have come from others that fluoridation deteriorates the brain or causes cancer. To combat such unscientific protestations we need facts. We must know more of the effect of food habits, water drinking habits, body weight and form, age, disease, and metabolic state on fluorine utilization in the body. The fact that Martin found that spinach cooked in the open pan absorbs much more fluorine than beets cooked in a pressure cooker may be more important than it appears to the casual reader.

Rogosa, Mitchell, and Wiseman (p. 682) have developed a selective medium for oral lactobacilli which they suggest is superior to tomato juice agar. It is easily prepared, inexpensive, and eliminates growth of most organisms other than lactobacilli. In comparing it with tomato juice agar they considered the results favorable. Of course, it is extremely difficult to reproduce lactobacillus counts even on repeated plates from the same sample. (Permar, D., Kitchin, P. C., Robinson, H. B. G. *J. D. Res.* **25**: 475, 1946; Dewar, M. R., *D. J. Australia* **21**: 509, 1949.) To eliminate this difficulty, the authors used reproductions within 100 per cent variation as acceptable and found that this occurred in 55 per cent of 122 samples from human beings and in 43 per cent of 156 hamster samples. The method, no doubt, eliminates many undesirable bacterial forms and molds but it should be evaluated against caries before standards for susceptibility and immunity may be established on this medium.

Nuckolls, Killian, and Frisbie (p. 690) present a well-illustrated description of early caries in decalcified sections of hamster molars. It is most interesting that they interpret their material as giving evidence that caries is initiated in the organic material when their method of preparation has removed the inorganic material and prevented observation of changes in that phase of the enamel. Descriptions of dynamic processes, such as dental caries, on the basis of limited observations on "still pictures" of treated tissues (fixed, dehydrated, decalcified) may lead to conclusions as erroneous as those of the blind men describing an elephant.

Besic (p. 708) shows artificial caries produced in three teeth. The carious lesions produced closely resemble those observed in clinical practice. Clinically and radiographically they are indistinguishable from such lesions. It has often been said by investigators, who have not bothered to review the literature, that dental caries has never been produced in vitro. This paper is another which demonstrates the production of such lesions and their similarity to clinical dental caries.

Ogilvie (p. 712) experimentally produced acute and chronic sodium fluoride intoxication in a group of animals. The studies of the salivary glands indicate that there are significant changes in these glands under the influence of sodium fluoride. Just what role these changes play in the problems of general and oral pathology is not yet clear. However, the fact that the fluorides do have effects on the cytological picture of the salivary glands strongly suggests that some of the influence of fluorides on dental caries may come through the secreted saliva. The complete evidence for this, of course, is not yet available but further investigation of the physiology as well as the cytology of the salivary glands under sodium fluoride administration is suggested.

Bartelstone (p. 728) has demonstrated the relatively rapid (1½-2 hours) penetration of radioiodine through intact enamel of teeth in vivo. It seems apparent that the iodine rapidly reaches the blood stream (as evidenced by thyroid radioactivity) and, probably by a direct route, the periodontium. This may be accepted as evidence of the penetrability of intact enamel in vivo. Its relationship to metabolism will probably remain for debate —those who look on enamel as a substance with metabolic activity citing this work as evidence for their viewpoint while those who

consider enamel a relatively inert cellular product considering only as evidence that the enamel acts as a permeable membrane. The evidence at hand, using a new research tool, establishes that intact enamel, in vivo, is permeable in relative short time periods.

Kupfer (p. 734) found that 34 of 36 patients with toxic goiter had one or more hypercementosed teeth. Using groups of comparable age from other hospital cases, he found only 6 of 36 patients with hypercementosed teeth and in a similar group of nonhospitalized patients he found only 7 of 36 patients with one or more hypercementosed teeth. Kupfer does not make any effort at the present time to explain the nature of this relationship between hypercementosis and toxic goiter.

Production of heat during cavity preparation is detrimental to the dental pulp. Many factors contribute to heat production in cavity preparation. Vaughn and Peyton (p. 737) have investigated the influence of some of these factors on temperature rise during cavity preparation. They find that with a fissure bur the temperature rises at speeds between approximately 1,100 and 11,000 r.p.m. and that the maximum temperature rises develop within ten seconds after the operation begins. As has been suggested from clinical studies and observations, the smaller the bur the lower the temperature rise produced. They also found that both increase in pressure and increase of speed of operation has a pronounced influence on the temperature rise developed in the tooth. This study is of importance to those who are interested in preservation of the dental pulp during cavity preparation, and that should include all dental operators.

Bush and Peyton (p. 745) report the effect of section size on mechanical properties of wrought gold wires. Oddly, such a study had not been undertaken previously. The authors found published results of proportional limits of various alloys unreliable for designed purposes under certain conditions.

STUDIES IN THE REGENERATION AND REATTACHMENT OF SUPPORTING STRUCTURES OF THE TEETH

II. Regeneration of Alveolar Process

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INTRODUCTION

A SERIES of studies on the repair of the periodontal structures following surgical removal of a section of alveolar bone and periodontal membrane is being made on dogs. The first paper reporting this work described a procedure whereby reattachment of the soft tissues to the tooth was attained.[1] This communication presents a further study of the reparative process in which both reattachment of the soft tissues to the tooth and a considerable regeneration of alveolar process have been observed.

Three tissues are required for the regeneration of the periodontal structures—cementum, bone, and fibrous connective tissue. Therefore, cementoblasts, osteoblasts, and fibroblasts are necessary for the reparative process. Fibroblasts, since they are always available for the repair of wounds, do not present any special problem, but cementoblasts and osteoblasts do. We know that osteoblasts make their appearance where bone is being formed, but where they come from and what is the cause of their development has been the subject of a great deal of controversy for nearly a hundred years. This is indicated by the many conflicting hypotheses on the causality and mechanism of osteogenesis. For example, according to one theory in the usual reparative process, osteogenic cells from bone itself rather than cells of ordinary connective tissue are utilized to form new bone. Another theory is that proliferating connective tissue cells anywhere in the body may be the site of bone formation. Whether or not bone is formed depends, according to this second theory, mainly on environmental factors.

In the light of these conflicting opinions, the origin of the cells for the reparative process in which we are interested may be important. Have the cells of granulation tissue arising from the connective tissue of the gingiva the potentiality to form osteoblasts, or must the sources of the cells be the alveolar process? In addition to osteoblasts, new cementoblasts are essential for the reattachment of the soft tissue to the tooth.

With these ideas in mind an examination of Fig. 1, a specimen from the study of reattachment after a healing period of 49 days, is of interest as a partial success was attained in promoting the desired type of repair.

It is evident that the surgically removed bone and periodontal membrane have been replaced by a tissue largely cellular in nature. Some of the cells near the cut edge of the alveolar process have differentiated to osteoblasts and formed bone, while others lying against the tooth became cementoblasts and

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formed cementum. However, while the formation of new cementum occurred over approximately three-quarters of the denuded tooth surface, only a small regrowth of the alveolar process took place. The failure of the majority of the replacement cells to differentiate to osteoblasts may have been the reason for this relative lack of bone growth. If this was the case, was the failure of the cells to differentiate to osteoblasts due to lack of potentiality for differentiation, or to a lack of the required environmental stimuli? The origin of the cells, whether from the connective tissue of the gingiva or from the bony margins of the wound, is not clear as yet. To decide between these two alternatives, an attempt was made to provide for these cells appropriate environmental stimuli of the type that in the previous study on reattachment had seemed to favor differentiation to osteoblasts. This was done by the use of autogenous bone grafts as described below.

Again in Fig. 1, cementum is seen to have been formed. The question arises, whether the new cementoblasts were formed by the cementoblasts at the margin of the wound proliferating along the tooth, or by differentiation of the replacement cells that came in contact with the tooth. If the latter supposition is correct, it would appear either that the tooth has some property that induces the cells to differentiate to cementoblasts rather than to osteoblasts, or the difference between these cells is one of position only. In either case it would appear that both bone and tooth structure have something to do with the differentiation of the replacement cells. The observation in Fig. 1, that differentiation to osteoblasts and cementoblasts occurred only near bone and tooth, respectively, would support this hypothesis. Accordingly, in the present study to test this premise, grafts of tooth structure chipped from the denuded tooth were mixed with the bone grafts in such a manner that more of the proliferating replacement cells would be in contact with these hard tissues.

The experimental procedures to be described were designed to test the hypotheses outlined above. The results have shown that even with cells of proved potentiality for differentiation to osteoblasts, no such differentiation takes place unless the required environmental stimuli are present.

It might be well to suggest here what would happen if, through lack of potentiality or environmental stimuli, no such differentiation of the replacement cells took place. Without new cementum there would be no connective tissue union with the tooth, the oral epithelium would proliferate apically in which case there would be no regeneration of alveolar bone. Thus it is obvious that an understanding of the factors concerned in the required cellular differentiation is basic to this problem of reattachment and regeneration of the alveolar bone.

PROCEDURE

The 2 upper canine teeth of adult dogs with healthy gingival tissues were used in this study. The procedure for the preparation of the 3 dogs whose sections are shown in Figs. 1, 2, and 3 was that described in the previous paper.[1] Briefly, by means of a disk stone a groove was cut into the tooth approximately 1 mm. from the gingival margin. This served as a point of reference from which

the level of the gingival sulcus could be measured. A gingival flap was made, exposing the overlying alveolar process, care being taken to detach the complete periosteum including the inner or cambium layer. Then a section of bone and periodontal membrane approximately 7 by 6 mm. was removed, exposing the tooth. The flap was sutured into place.

The procedure for the remaining specimens was similar with the exception that the space created by the removal of the section of the alveolar bone and periodontal membrane was filled with bone chips approximately 2 by 3 by 1 mm. in size, before the flap was sutured into place. In most cases the alveolar process removed was used for the grafts, but in a few cases the grafts were obtained by removing part of the alveolus from a neighboring tooth. In addition, grafts of tooth structure obtained by chiselling off small chips of the exposed root were mixed with the bone chips in some of the specimens. The animals were sacrificed at various time intervals ranging from 12 to 130 days, and blocks were removed for sectioning. Paraffin sections were made after fixation in 10 per cent formol-saline, decalcification in 5 per cent formol-nitric acid, and staining with hemotoxylin and eosin.

OBSERVATIONS

In these studies a wavy irregular outline of the root surface, Figs. 1 and 3, indicates an area of root that has been denuded. It follows then that when viewing a section, alveolar process opposite such a root surface must be new bone. This offers a convenient method of identifying replaced bone up to at least a year and a half after the production of the lesion.

Figs. 2, 3, and 1 are very low power photomicrographs of specimens after 14, 22, and 49 days of healing, respectively. Healing occurred without the use of grafts. The magnification is the same in Figs. 1-6.

In Fig. 2, new trabeculae of bone can be seen apparently growing from the cut edge of the alveolar process at the base of the wound. An examination of Figs. 3 and 1 indicates that up to the forty-ninth day little extension of the osteogenic process had occurred, the amount of new bone formed being negligible. Figs. 4, 5, and 6 are photomicrographs after 14, 21, and 30 days, respectively, from experiments in which grafts were used. In Fig. 4 trabeculae of new bone can be observed to have been formed a much greater distance from the cut edge of the alveolar process than was the case on the the fourteenth day where grafts had not been used (Fig. 2). In Figs. 5 and 6, with a time interval comparable to that of the specimens in Figs. 3 and 1, the extent of the formation of new bone is so great that the fields depicted are not large enough to show all of it.

It appears then that the distance which new trabeculae grow from the old bone is much greater when grafts are present.

Figs. 1 to 20.—Key to abbreviations in illustrations: A = artefact (separation believed to have occurred during the preparation of the sections); AB = alveolar bone; BG = bone graft; C = cementum; CB = cementoblast; CC = cementoclast; D = dentin; DG = dentin graft; DF = dentinal fibril; EL = empty lacunae; GF = gingival flap; J = junction of old and new attachments; NB = new bone; NC = new cementum; NO = new osteocytes; NT = new trabeculae; OB = osteoblast; OC = osteoclast; PM = periodontal membrane; R = resorption; RC = replacement cells; T = tooth; TOL = thickened osteogenic layer.

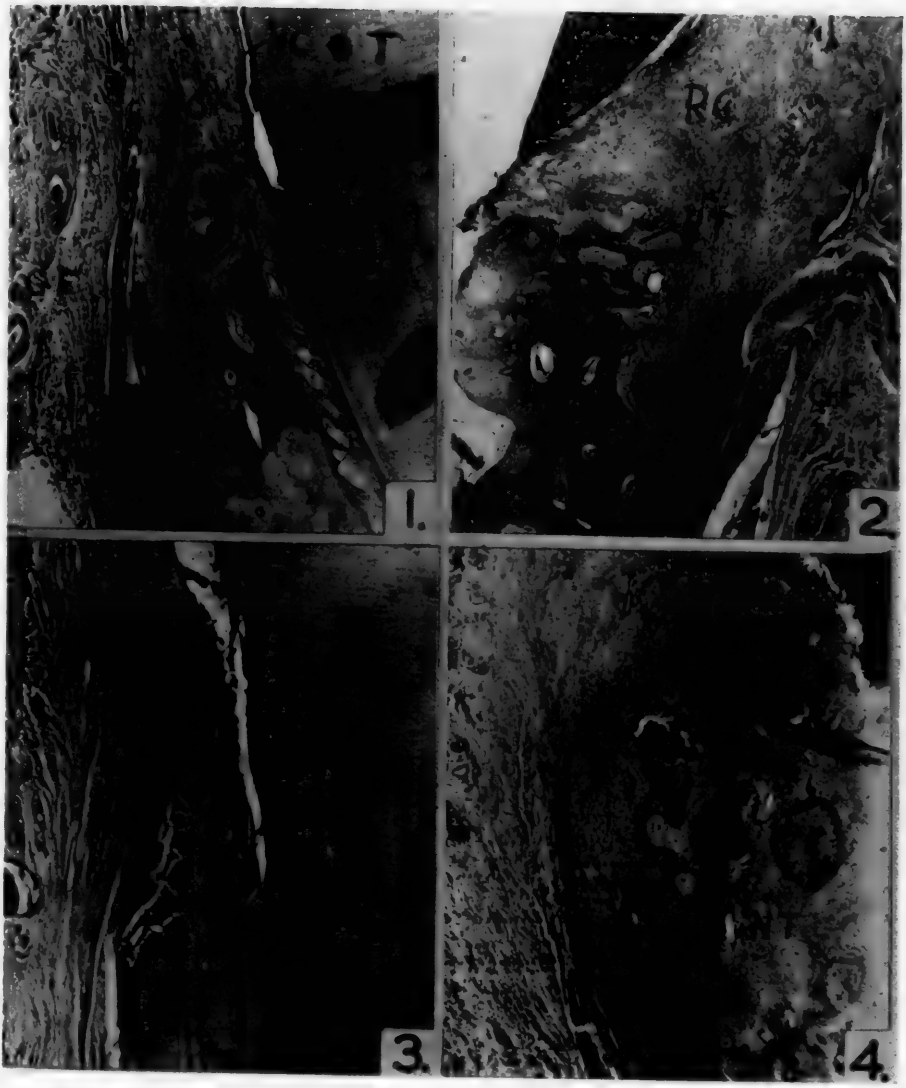

Fig. 1.—Healing without grafts, 49-day specimen, showing reattachment.
Fig. 2.—Healing without grafts, 14-day specimen, showing new bone.
Fig. 3.—Healing without grafts, 22-day specimen, showing new bone.
Fig. 4.—Healing with grafts, 14-day specimen, showing increased amount of new bone.

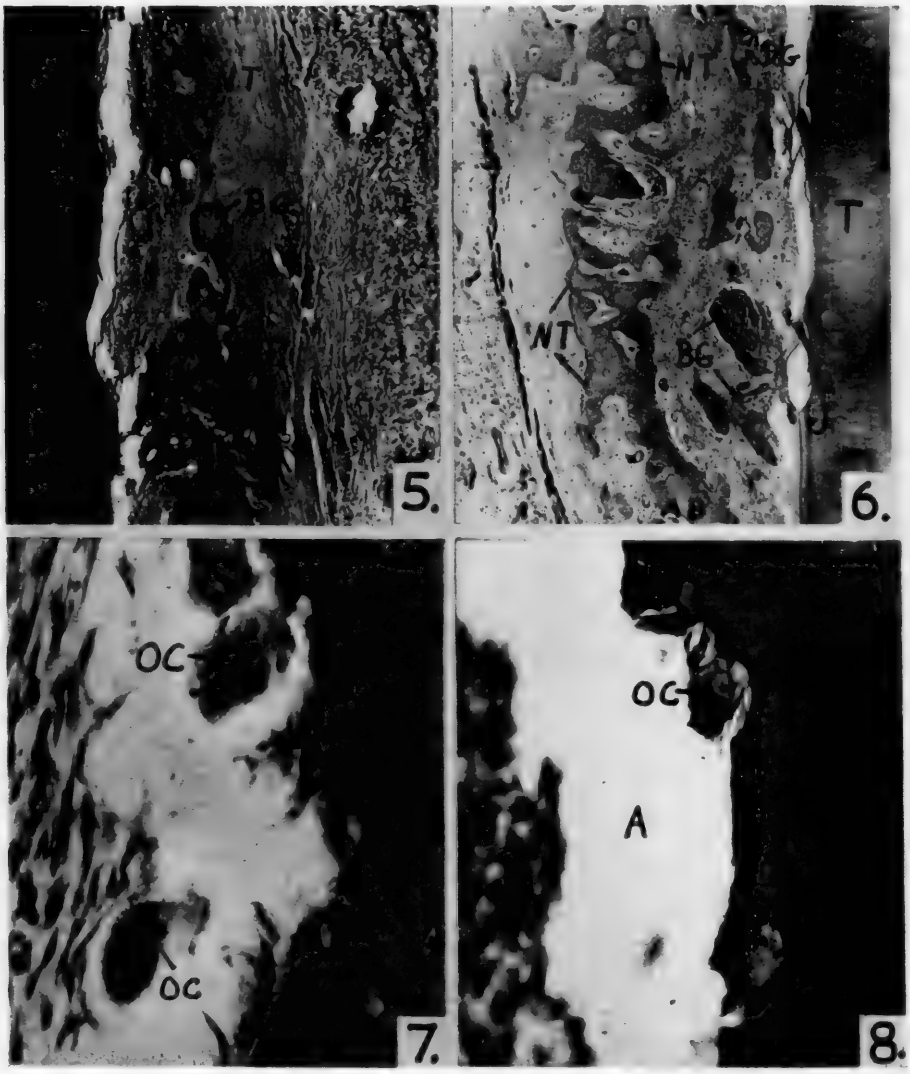

Fig. 5.—Healing with grafts, 21-day specimen, showing increased amount of new bone.
Fig. 6.—Healing with grafts, 30-day specimen, showing increased amount of new bone.
Fig. 7.—Specimen of 17 days showing osteoclastic resorption of alveolar process at margin of wound.
Fig. 8.—Specimen of 14 days showing resorption of denuded root surface.

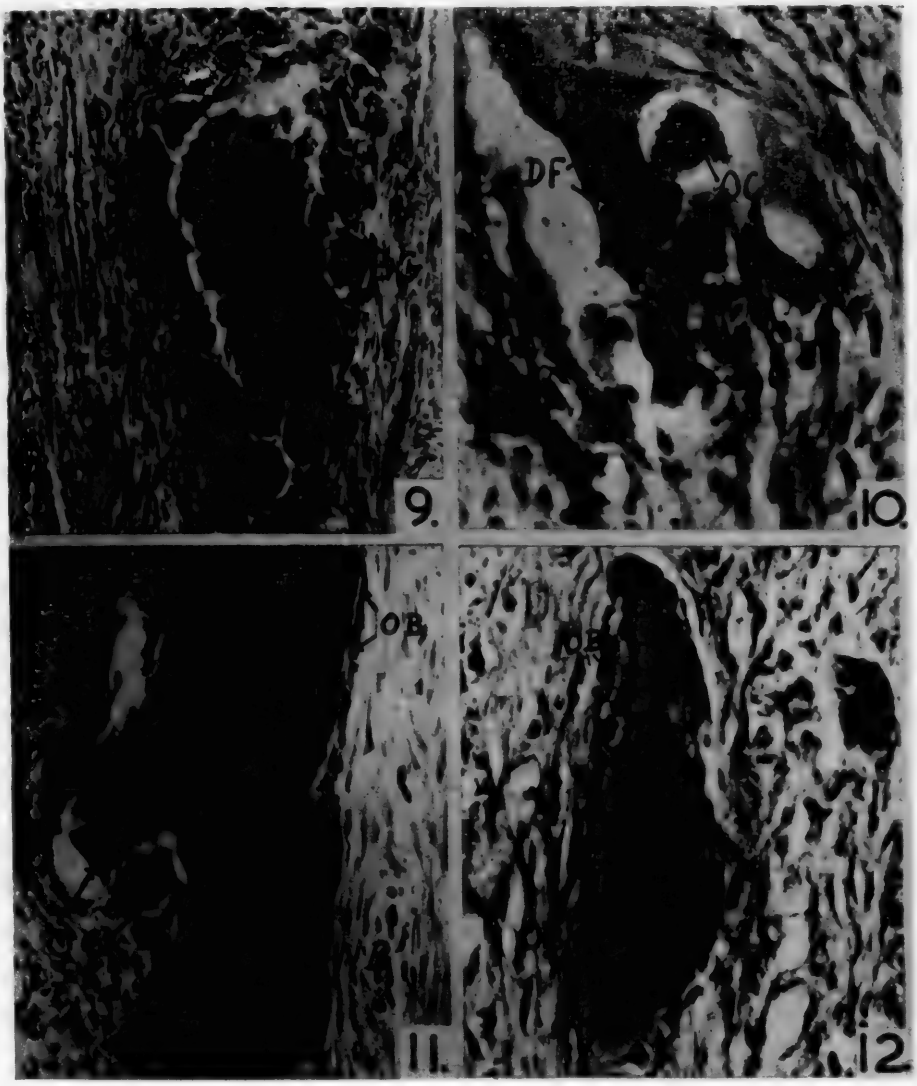

Fig. 9.—Specimen of 17 days showing osteoclastic resorption of a bone graft.

Fig. 10.—Specimen of 14 days showing resorption of a graft of tooth structure.

Fig. 11.—Specimen of 17 days, bone graft at transitional stage, showing both resorption and apposition.

Fig. 12.—Specimen of 21 days showing early apposition on graft of tooth structure.

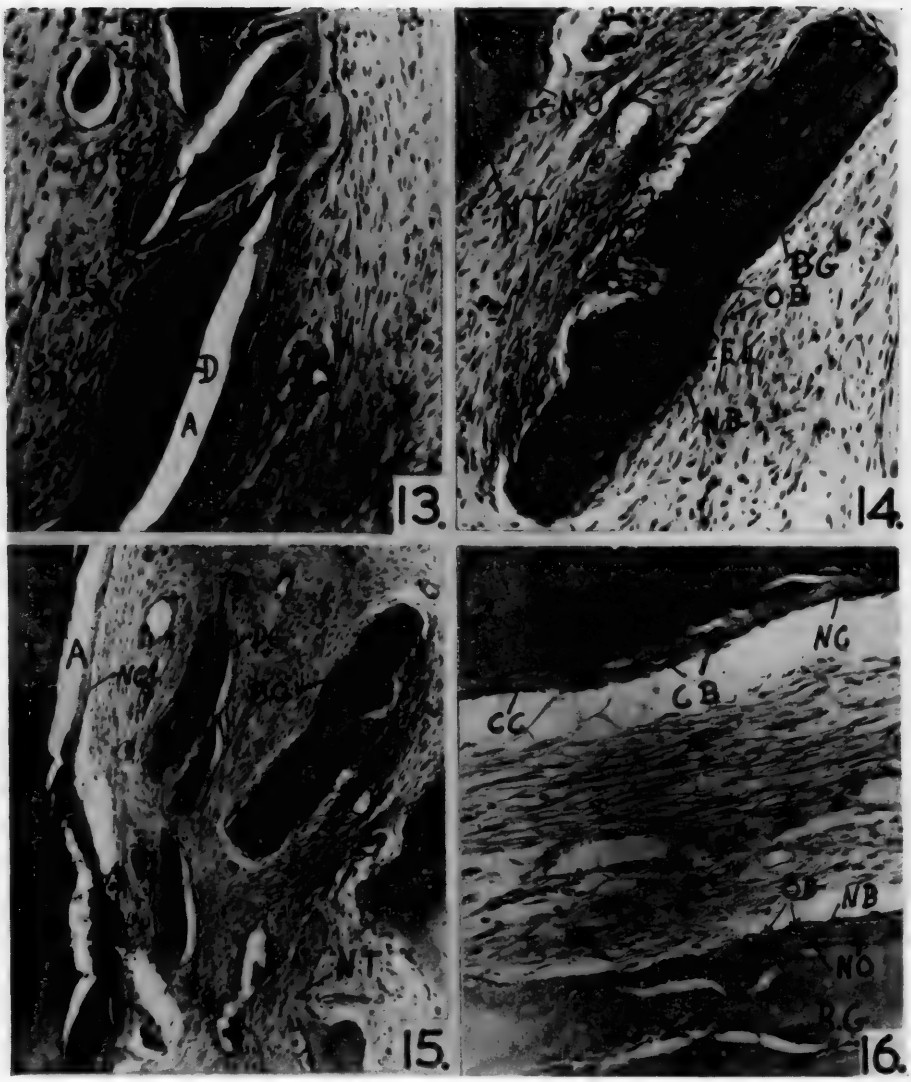

Fig. 13.—Specimen of 30 days showing apposition on graft of tooth structure.

Fig. 14.—Specimen of 30 days showing apposition on bone graft and part of trabecula of new bone.

Fig. 15.—Specimen of 30 days showing apposition on denuded root, on grafts of tooth structure and bone, and part of trabecula of new bone.

Fig. 16.—Specimen of 35 days. Compare apposition on bone graft with apposition on tooth.

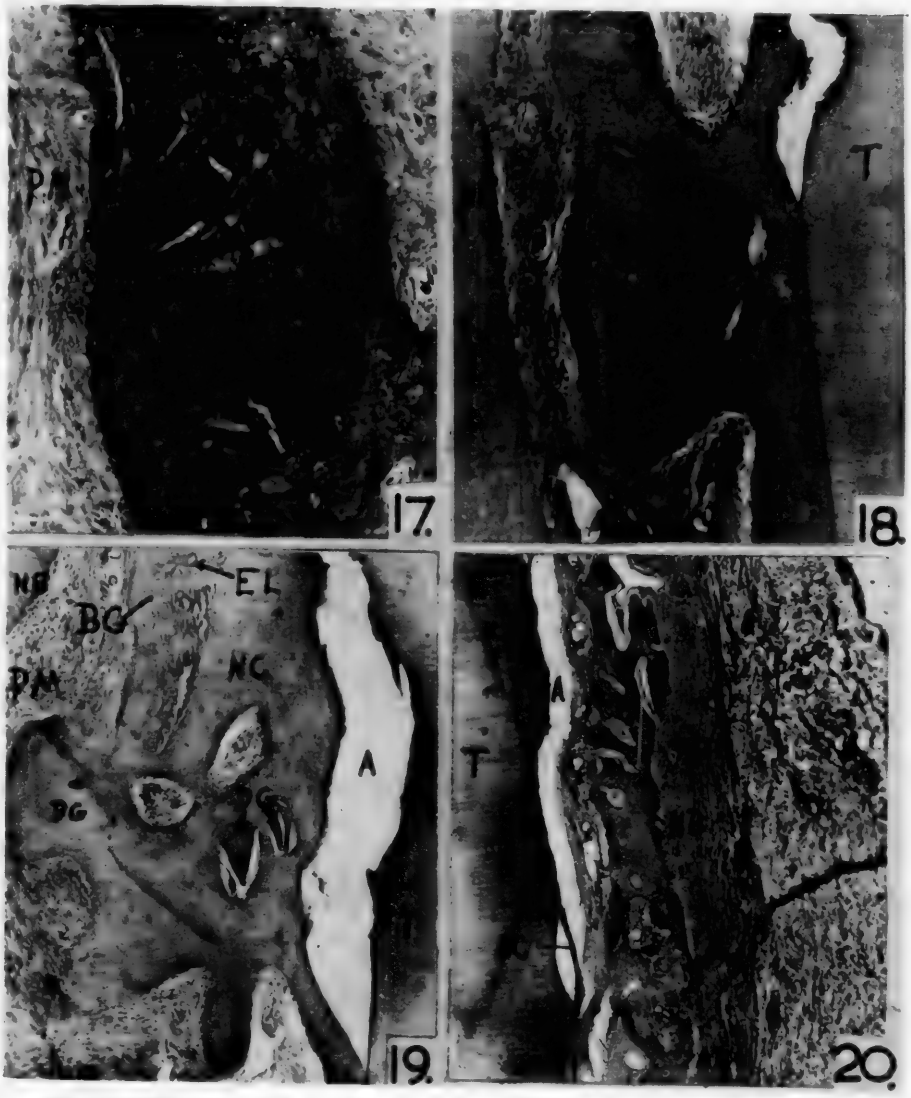

Fig. 17.—Specimen of 43 days showing empty lacunae in new bone, suggesting a remnant of bone graft.

Fig. 18.—Specimen of 120 days showing bone graft embedded in new cementum.

Fig. 19.—Specimen of 120 days showing grafts of both bone and tooth structure embedded in new cementum.

Fig. 20.—Specimen of 55 days showing reattachment and regeneration of alveolar process.

The low magnifications used in Figs. 1 to 6 were adopted in order to show as large a field as possible, but in order to study the details of the reparative process, higher magnifications (Figs. 7 to 19) were necessary. Figs. 7 to 10 illustrate resorption of the cut edge of the alveolar process, the root of the tooth, and grafts of bone and dentin respectively, with a healing period of from 14 to 17 days. Up to the seventeenth day, with the exception of the growth of new trabeculae, the picture is one of resorption.

About the seventeenth day the osteoclasts disappear, and osteoblasts begin to appear on the surfaces which up to then showed only resorption, and the picture changes to one of regeneration and apposition. Fig. 11 (17 days) is a high power magnification of a graft in the transitional period. At the left, osteoclastic resorption is still occurring along the central part of the margin of the graft. Along the right border and part of the left, osteoblasts can be seen lined up in their characteristic manner. Fig. 12 (21 days) is a high power photomicrograph showing differentiation of osteoblasts and early apposition around a graft of tooth structure. This can be more clearly seen in Fig. 13 (30 days), where apposition is seen around a dentin-cementum graft and a graft of dentin only. Note the newly formed osteocytes. Fig. 14 is from the same specimen showing apposition on a bone graft. The new bone in the upper left hand corner is part of a new trabecula. The new trabeculae seem to grow up between and around the grafts but without intimate contact with them. The contrast between the deep staining osteocytes in the trabeculae and the empty lacunae characteristic of the bone grafts is quite clear. In Fig. 15 (30 days), a low power magnification shows apposition on the tooth, on grafts of tooth structure and on grafts of bone; also on the right side there is a large well-formed new trabecula that has grown beside the grafts without actually touching them.

It appears that when grafts are used, new bone is laid down as new trabeculae and also by apposition on the grafts. In these studies new trabeculae growing from the cut edge of the alveolar process could be observed after 12 days, the time at which the earliest specimens were studied. Gordon and Ham[2] observed new trabeculae at the edge of an experimental defect in long bones as early as 6 days after the operation. In our studies apposition on the grafts was not observed before the sixteenth day.

In Fig. 16 (35 days), apposition on the bone graft at the bottom can be compared with apposition on the tooth. The time interval for apposition, and the amount deposited appear to be of the same order.

Later, around the fortieth day, the new trabeculae and the new bone laid down on the grafts seem to fuse together and it is difficult to determine the fate of the grafts. No grafts of tooth structure or bone could be clearly distinguished at this time in the newly formed bone. However, in Fig. 17 (43 days) the presence of empty lacunae in new bone suggests a remnant of a graft.

It seems probable that bone grafts are gradually absorbed and replaced by new bone, for in Fig. 18 (120 days) there is no evidence of the presence

of grafts in the newly formed alveolar bone on the left, even though grafts were present in the area, as indicated by the fact that in new cementum formed at the same time, a bone graft can be clearly distinguished. Cementum is normally an unvascularized tissue, which probably explains the presence of the graft after 120 days. In this specimen the material laid down on a bone graft has now become cementum. However, in Fig. 19, a field from the same case showing both dentin and bone grafts embedded in new cementum, it would appear that this mass of cementum is gradually being remodelled to a more normal physiological form.

Fig. 20 (55 days) shows, under a very low magnification, a considerable regeneration and reattachment of the supporting structure of the tooth.

DISCUSSION

The presence of calcified tissues appears to be a factor in the differentiation of osteoblasts. In these studies, as the differentiation occurred only in the regions where calcified tissue was being resorbed, it may well be that it is the presence of resorbing, calcified tissue that provides the stimulus in some as yet unrecognized way for the differentiation of these cells.

No essential difference was observed between the effect of grafts of bone and grafts of tooth structure. Dentin contains protoplasmic extensions of the odontoblasts; otherwise it is acellular. This observation suggests that the osteogenic effect of grafts is due less to their cellular content than to the calcified intercellular material.

There appears to be little if any difference between the osteoblasts and cementoblasts in the reparative process. No difference could be detected between the material laid down on the grafts which later became bone and that which was deposited on the tooth. The presence of bone grafts in cementum supports this concept, for it indicates that the osteoid tissue laid down on the grafts may be incorporated in either cementum or in bone.

Grafts seemed to encourage osteogenesis by stimulating the growth of new trabeculae and by acting as new islands of ossification. When grafts are used, the pattern of bone growth may be likened to an advancing army with groups of paratroopers being dropped ahead of the main body. Besides facilitating the advance of the main body, each group of paratroopers presents an island of conquered territory which later, under favorable conditions, becomes consolidated with that of the main army.

The origin of the cells in the repair process, whether from the connective tissue of the gingiva or the bony margins of the wound is still not proved. However, the evidence favors the view that these cells came from the bony margins of the wound. A characteristic thickening of the inner layer of the periosteum near the cut edge of the alveolar process indicates proliferation of these cells following the operation. The tissue that replaced the removed bone looked similar to the tissue in the thickened area of the periosteum. Again, the trabeculae of new bone appeared to grow out from the old bone, which suggests that the cells concerned came from the same source. However, further work to fix more definitely the origin of the replacement cells will be carried out.

The formation of the cementoblasts along the dentin of the denuded tooth, and of the osteoblasts around grafts of dentin, suggests that these cells differentiated from the replacement cells that came into contact with them during the healing process. However it is quite possible that some of the cementoblasts and endosteal cells present on the graft survived and contributed to osteogenesis. But as cementoblasts were formed equally well whether the tooth was scaled or not, a procedure which tends to remove any remaining cementoblasts, it appears probable that the cells of the transplants do not contribute greatly to the osteogenic processes.

More of the replacement cells differentiated to osteoblasts and formed bone when grafts were used than when healing occurred without grafts. This suggests that many of the replacement cells which did not differentiate when grafts were not present had the potentiality to become osteoblasts. Thus, it would appear that even with cells of known potentiality to become osteoblasts appropriate environmental stimuli are necessary for differentiation. This may be an explanation for the observation in the former study on reattachment that although little new bone was formed, reattachment by means of new cementum occurred over approximately three-quarters of the denuded root.

CONCLUSIONS

1. In dogs, following the surgical detachment of the soft tissues and removal of a section of bone and periodontal membrane, considerable soft tissue reattachment and regeneration of alveolar process have been attained.

2. Resorption of calcified tissue seems to be a factor in the differentiation of osteoblasts and cementoblasts.

3. It appears that the osteogenic effect of grafts is due less to their cellular content than to the calcified intercellular material.

4. No difference could be detected histologically between the osteoid tissue laid down on grafts and the new cementoid tissue on the tooth.

5. The origin of the cells in the reparative process is still not clear but the evidence favors the view that the majority of the cells came from the bone rather than from the connective tissue of the gingiva.

6. Without the appropriate environmental stimuli cells, with the potentiality to become osteoblasts, did not differentiate to osteoblasts.

7. A theory of the mechanism of the repair of bone with and without the use of grafts has been advanced.

The histological sections were prepared by Mr. D. H. Beaton; the photomicrographs by Dr. W. Stanley Hartroft.

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EFFECTS OF ACUTE FEBRILE DISEASES ON THE PERIODONTIUM OF RHESUS MONKEYS WITH REFERENCE TO POLIOMYELITIS

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A GREAT number of systemic diseases have been listed as contributing to, or even causing the occurrence of periodontal disease.[1, 4, 23] Acute febrile diseases have been considered to be important in the pathogenesis of periodontal disease, especially since Gottlieb[11, 12] described diffuse atrophy of the alveolar bone in a young man who died of influenza. Talbot[41] noted as early as 1899 an association between eruptive fever and impairment of the periodontal tissues. Acute febrile diseases have been mentioned as part of the complex of systemic diseases which might cause periodontal disease.[3, 5, 29] Patients frequently recall soreness of the teeth, gingival tenderness, and hemorrhage which have occurred during an attack of acute febrile disease. They therefore associate elevation of temperature as an initiating factor in periodontal disease.

No studies have been reported in which the gingival conditions were observed before, during, and after attacks of acute febrile disease. The theory of a relationship between this group of diseases and periodontal disease has therefore been built upon the history obtained from patients, clinical assumption, and mainly on a vague general concept of an influence of systemic disease on the metabolism and vitality of the periodontal tissues.[21, 38] Little is known of the nature of specific tissue vitality, tissue response, and tissue resistance related to systemic disease,[7] thus a general conclusion pertaining to the periodontium is of questionable value. Experimental investigations of the periodontal tissues during the course of acute febrile disease have not been reported previously.

The opportunity for an investigation of this problem was provided at the University of Michigan School of Public Health where work was done on experimentally-produced poliomyelitis in rhesus monkeys. This disease produced a high fever of a limited duration. In rhesus monkeys the morphology and physiology of the periodontium and the masticatory movements are essentially the same as in human beings. It has furthermore been established that the oral bacterial flora of rhesus monkeys is the same as the flora of the human mouth.[2] The monkeys were kept on a well-balanced human diet.

Nineteen rhesus monkeys were utilized as experimental animals and seven other healthy monkeys, exposed to the same environmental and dietary conditions, were used as controls. Only monkeys which at necropsy had no

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evidence of tuberculosis and were comparatively free of animal parasites were included in this report. The pre-experimental gingival condition varied from normal to severe gingivitis. Color photographs and gingival biopsies were taken before the poliomyelitis virus inoculation. The temperature of each animal was recorded daily, and at the same time their mouths were inspected. The monkeys lived from five to sixteen days following the virus inoculation and had high temperatures for from one to ten days (Table I).

TABLE I

MONKEYS WITH POLIOMYELITIS*

MONKEY NO.	AGE (YEARS)	CLINICAL DIAGNOSIS GINGIVA BEFORE THE EXPERIMENT	DAYS FROM POLIOMYELITIS INOCULATION TO DEATH	DAYS WITH FEVER TEMP. ABOVE 104° F.	MICROSCOPIC PERIODONTAL FINDINGS
1898	3½	Mild gingivitis	16	4	Mild chronic gingivitis
1989	2½	Normal	9	4	Normal gingiva
1988	3	Normal	12	4	Mild chronic gingivitis
1987	2½	Mild gingivitis	9	5	Mild chronic gingivitis
1986	2½	Normal	16	4	Normal gingiva
1985	2½	Normal	9	4	Mild chronic gingivitis
1984	2½	Mild gingivitis	16	4	Mild chronic gingivitis
1983	2	Mild gingivitis	7	3	Mild chronic gingivitis
1982	2	Mild gingivitis	7	2	Mild chronic gingivitis
1981	3	Mild gingivitis	7	1	Normal gingiva
1927	2	Mild gingivitis	14	10	Mild chronic gingivitis
1932	2	Mild gingivitis	10	9	Mild chronic gingivitis
1928	2	Normal	10	5	Very mild chronic gingivitis
1933	2	Gingivitis	6	2	Mild chronic gingivitis
1919	3½	Mild gingivitis	10	8	Mild chronic gingivitis
1916	2	Normal	10	8	Mild chronic gingivitis
1920	3	Mild ulcerating gingivitis	8	4	Mild erosive gingivitis
1912	2	Mild ulcerating gingivitis	5	2	Mild erosive gingivitis
1910	3½	Normal	5	4	Normal gingiva

*All dental roentgenograms were negative. No clinical nor histological periodontal changes during the disease.

When the animals reached a moribund stage they were sacrificed and a necropsy was performed. Gross findings were recorded, photographs and dental roentgenograms taken, and models of their teeth were made. Specimens for microscopic study were taken from jaws, teeth, temporomandibular joints, mesial end of one tibia, and from the following visceral organs: lung, peribronchial and mesenteric lymph nodes, stomach, liver, kidney, spleen, adrenals, and pancreas. Hematoxylin and eosin stains of sections were utilized for examination of all of the tissues. Heidenhain's modification of Mallory's connective tissue stain was used to study sections of jaws and joints to determine the presence of changes in collagen fibers. Such chances, if present, would possibly be helpful in an attempt to explain eventual lowered periodontal resistance as the result of disease with acute high fever. The seven control animals were sacrificed and examined in the same way as the experimental animals.

FINDINGS AND INTERPRETATIONS

The case reports are summarized in Table I. Visceral changes typical of high fever were observed in all of the experimental animals: acute passive congestion of the lungs and spleen, degenerative fatty infiltration of the liver (verified by fat stain), cloudy swelling of the kidneys, catarrhal gastritis and colitis, and edema of peribronchial and mesenteric lymph nodes were present. Spinal cord sections showed evidence of poliomyelitis.

Gingival changes could not be observed clinically during the experimental period, nor did histopathologic examination of specimens, taken before and at the ultimate stage of the disease, show structural changes in the periodontium. A slight increase of materia alba on the teeth was observed in the terminal stage in a few animals. An additional number of monkeys with

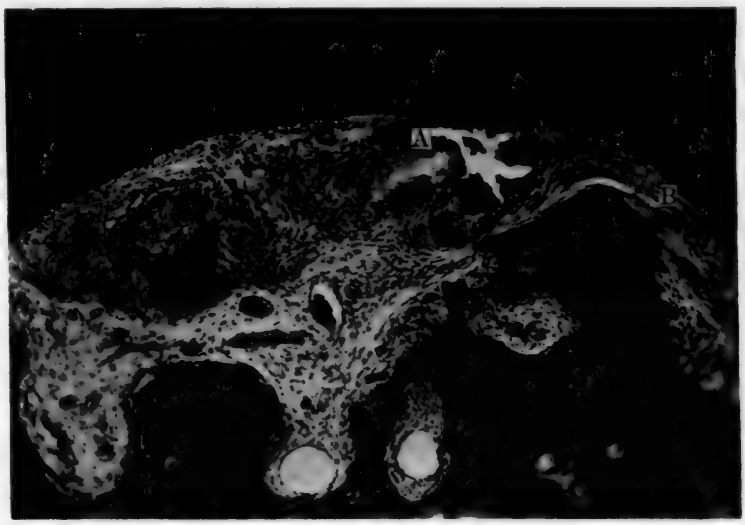
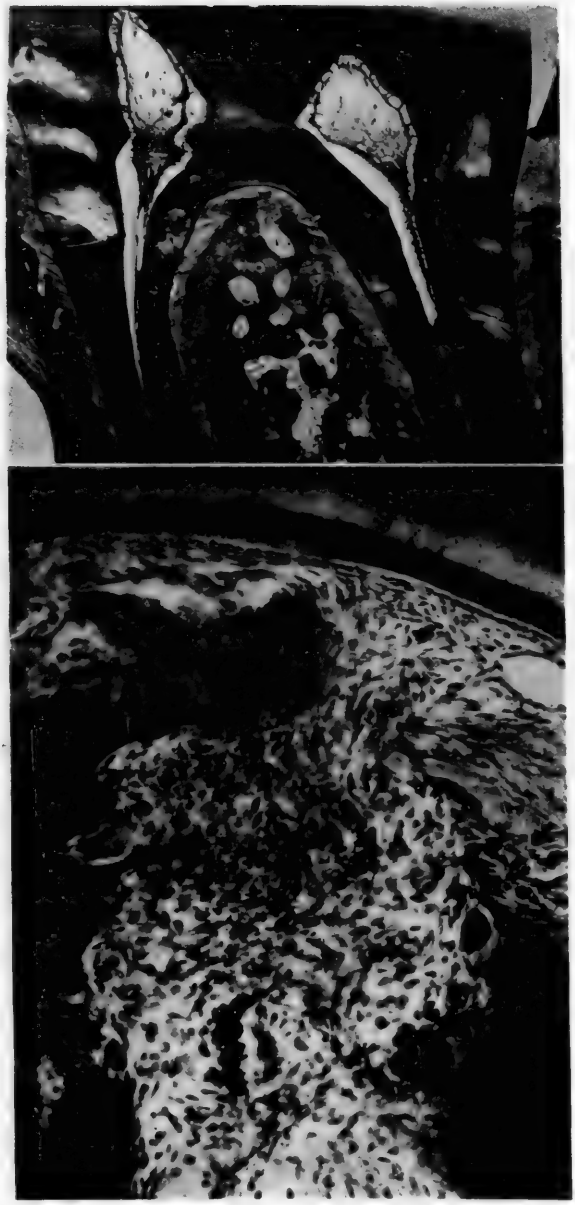


Fig. 1.—Monkey No. 1919. First molar (orig. mag. ×100). Recent trauma. Hemorrhage (*A*) and necrosis (*B*). Evidence of resorption and repair in other areas.

severe poliomyelitis were examined to clarify this observation, but the increase in soft debris coating the teeth was not a constant finding and the difference in quantity was slight, and therefore a definite statement cannot be made as to the significance of this observation.

The self-cleansing of the teeth by mastication of food; the flow of saliva; and the movement of the tongue, lips, and cheeks is of utmost importance in maintaining the oral hygiene, and thereby the health, of the gingival tissues. In severe febrile diseases the salivation is decreased due to dehydration, the food intake is lowered (requiring less chewing action) and other movements of the jaws are markedly decreased—also the personal oral hygiene is often neglected in advanced stages of systemic disease. This would all be conducive to increased accumulation of materia alba on the teeth, causing a

A.



B.

Fig. 2.—*A*, Monkey No. 1910. First molar. Small area of trauma in bifurcation. *B*, High magnification (orig. mag. ×120) of *A*. Resorption of alveolar bone, degenerating red blood cells from previous hemorrhage. Young granulation tissue.

gingivitis or aggravating an existing periodontal disease. Upper respiratory disease with high temperature frequently causes nasal obstruction and mouth breathing, resulting in gingivitis and pulpal hyperemia. Maxillary sinusitis associated with upper respiratory infection causes "sore teeth." Increased nervous tension is often observed in the terminal stage of severe illness and is manifest by an increased tendency for clenching of the jaws (Karolyi effect). The traumatic injuries to the pulp and the periodontal membrane from this process will cause the traumatized tooth to feel sore. These local factors are discussed here because they offer a plausible explanation for the claimed relationship between febrile diseases and periodontal disturbances.

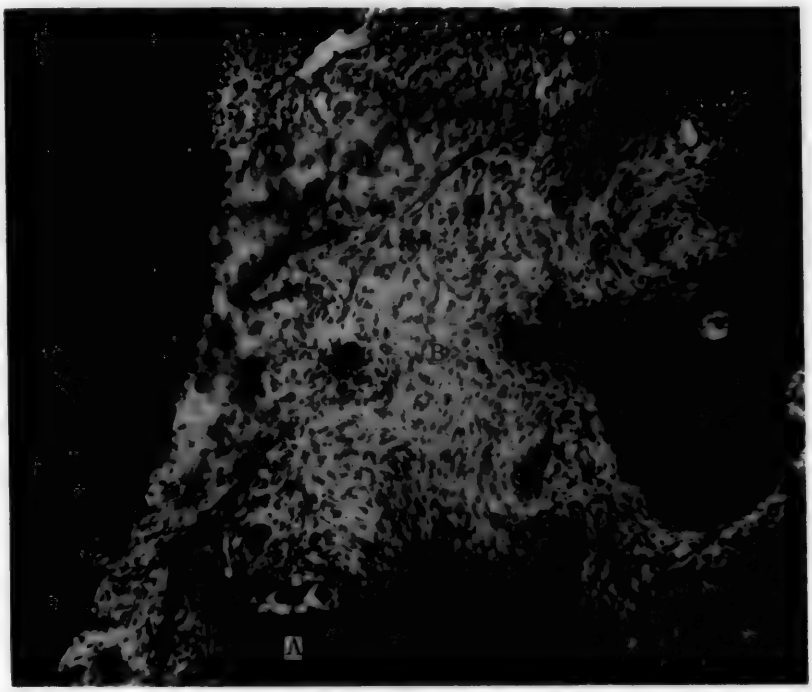
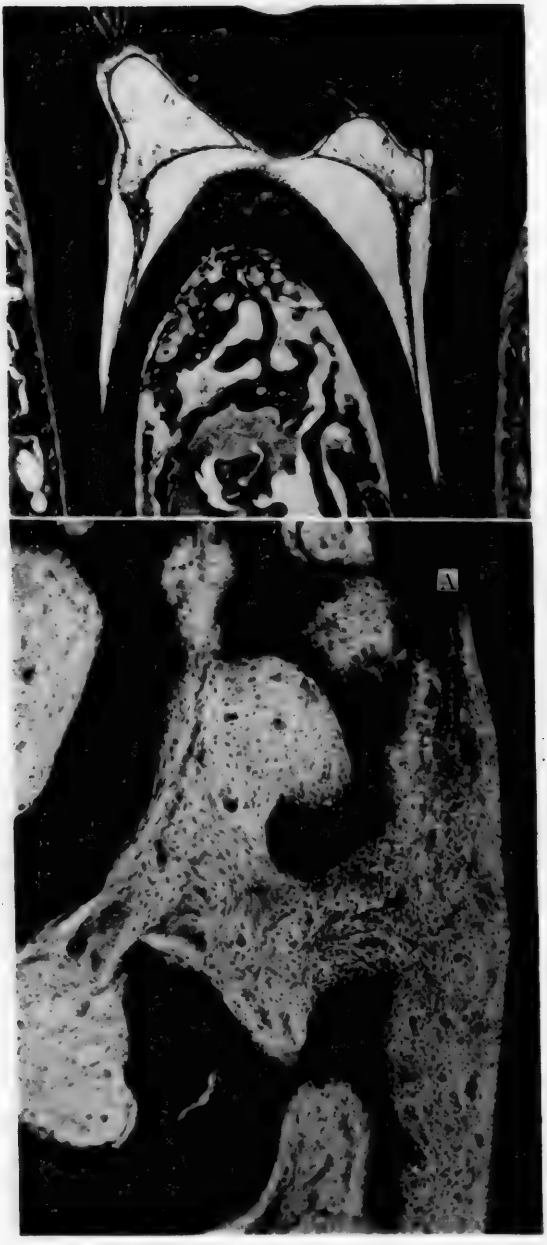


Fig. 3.—Control monkey. First molar (orig. mag. ×120). Resorption of alveolar bone (*A*). Replacement of lost bone and periodontal membrane by vascular loose connective tissue without functional orientation (*B*). Histologically resembling periodontosis.

One of the most important and extremely varying local factors is the change in performance and efficiency of the personal oral hygiene during a period of severe disease. The exclusion of this factor in experimental animals increases the significance of the findings pertaining to systemic factors as a possible cause of periodontal disease.[44]

Various stages of traumatism were observed in jaw sections from monkeys in the experimental and control groups (Figs. 1, 2*A* and *B*, and 3). A common location for these traumatic lesions was the inter-radicular space of the first molars, indicating an axial force. The lesions, conformed histologi-

A.



B.

Fig. 4.—*A*, Monkey No. 1916. First molar. Evidence of extensive inter-radicular trauma. Hemorrhage. Calcific deposits, resorption of bone, and fibrosis of marrow spaces. Widening of the periodontal spaces. Widening of periodontal space in the cervical region. *B*, High magnification of *A* (orig. mag. ×100). Recent trauma (*A*). The rest of the picture shows healing stage of traumatism, resembling periodontosis.

cally to the description in the literature of traumatic periodontal injuries.[8, 14-16, 27, 28, 32-34, 37, 39, 40] Thrombosis, hemorrhage, degeneration, necrosis, bone and root resorption, calcific deposits, and hyalin changes constituted the early manifestations[6, 22] (Figs. 1, 2*B*, 4*A*).

The healing stage of traumatic lesions in numerous areas showed features which resembled the commonly accepted picture of "diffuse atrophy of the alveolar bone" or periodontosis.[9, 10, 13, 17, 24, 31, 35, 42, 43, 45] A widespread resorption of alveolar bone, sometimes extending into the supporting bone, was observed (Figs. 3 and 4*A*). Fragments of degenerating periodontal fibers were seen in loosely arranged vascular granulation tissue which extended into the adjacent marrow spaces (Figs. 3, 4*B*, and 5). No difference in susceptibility to these lesions was noted in comparing experimental and control animals. A section of a human tooth showing the same type of traumatic periodontal changes is included for comparison (Fig. 6).

Fig. 5.—Tuberculous monkey. First molar (orig. mag. ×120). Repair following traumatic injury. Remains of old fibers in loosely arranged vascular granulation tissue.

It has been suggested by Häupl and others[18-20, 25, 30] that the histopathologic findings which Gottlieb, Orban, Weinmann and others[12, 13, 17, 31, 35, 38] have described as being pathognomonic of periodontosis are manifestations of occlusal trauma. In my investigation of the periodontium of rhesus monkeys these traumatic changes resembling degeneration have been observed in monkeys suffering from severe tuberculosis (Fig. 5) and alloxan diabetes, as well as in

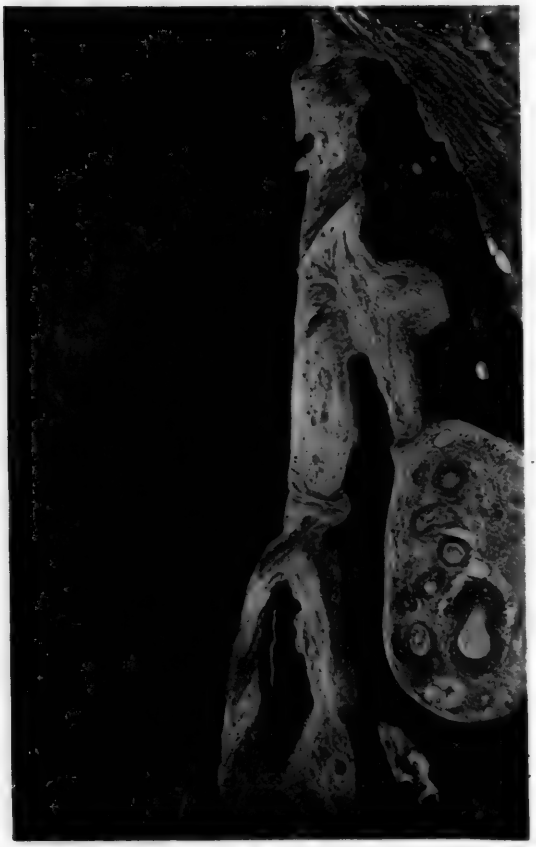


Fig. 6.—Human lateral lower incisor (orig. mag. ×65). Severe traumatic occlusion with cemental tears. Only small remaining bundles of periodontal fibers. Most of the periodontal space filled with loose unorganized connective tissue extending into the adjacent marrow spaces. Well-developed alveolar crest fibers.

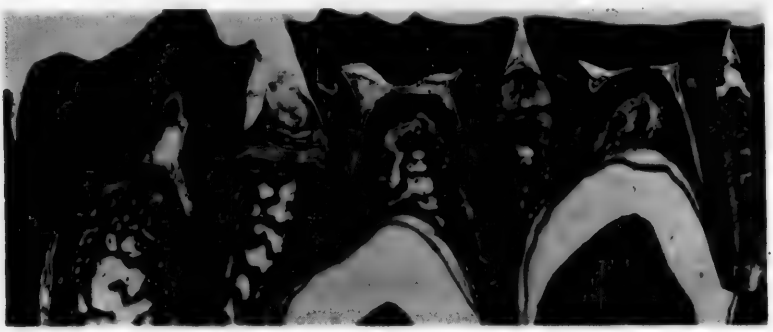


Fig. 7.—Monkey No. 1910. Various stages of root resorption. Inter-radicular and cervical evidence of traumatism. Gingivitis between the deciduous teeth.

the control animals. If the changes had not been observed simultaneously in the experimental and in the control animals there would have been a possibility of falsely interpreting the periodontal changes as due to the systemic diseases.

Deciduous teeth in various stages of root resorption showed traumatic lesions both in the inter-radicular and cervical region (Figs. 7, 9*A*, 9*B*). As a group the deciduous teeth showed more advanced gingivitis than the permanent teeth (Fig. 7) and the extent of the gingival inflammation appeared to increase with decrease in the length of the remaining roots (Fig. 8). This observation indicated that the increase in traumatic periodontal damage in teeth with short remaining roots contributed to an increased gingival inflammation by disturbing the blood supply and tissue metabolism, thereby lowering the resistance to infection and other local irritants.

Fig. 8.—Extent of gingival inflammation increased with decreasing length of the remaining roots. Trauma has led to fracture of mesial root of first deciduous molar and periodontitis.

Special stains for collagen proved to be of limited value in the detail study of collagenous fibers because these stains are influenced by factors which cannot be satisfactorily standardized in jaw sections containing teeth. Several sections from experimental and control animals showed artifacts closely resembling the fragmentation and degeneration of collagen fibers which have been described as being characteristic of periodontal changes seen in systemic disease.[36] A large number of sections from each specimen should be stained before an evaluation is attempted.

CONCLUSIONS

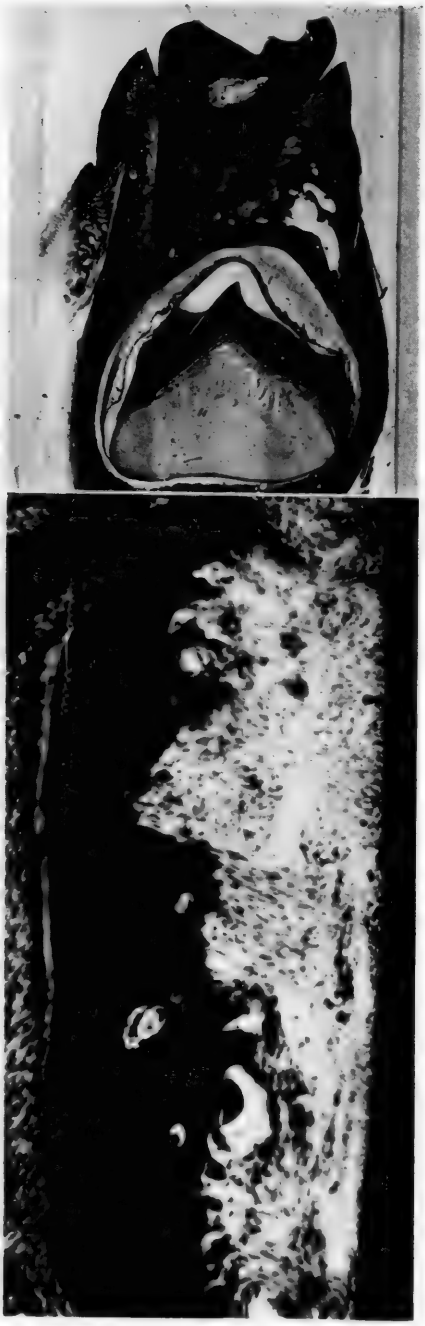
1. No changes attributable to poliomyelitis were observed in the periodontium of nineteen rhesus monkeys having the disease.

2. Special connective tissue stains did not reveal any structural changes in the collagenous fibers in joint or periodontal tissues of monkeys with severe poliomyelitis.

3. An association was noted between accumulation of debris on the teeth and gingivitis.

4. Gingivitis was observed more frequently and found to be more severe around deciduous than permanent teeth.

A.



B.

Fig. 9.—*A*, Monkey No. 1982. Active resorption of alveolar bone. *B*, High magnification (orig. mag. ×105) of *A*. Osteoclastic activity. Loosely arranged connective tissue constituting the periodontal membrane.

5. An inverse relationship was observed between the length of the root of the deciduous teeth and the degree of gingival inflammation.

6. The increase in incidence and severity of periodontal diseases which has been claimed to accompany febrile diseases is possibly due to increased local irritation, mainly because of poor oral hygiene, during the period of systemic illness.

7. The healing stage of certain traumatic lesions resembled the description of diffuse alveolar atrophy or periodontosis.

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LYMPHOCYTES IN THE EPITHELIUM OF THE HEALTHY GINGIVA

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LYMPHOCYTES, in small numbers, are conceded by most investigators to be normal constituents of the connective tissue. However, there is not unanimity of opinion in regard to the meaning of the presence of this slight lymphocytic infiltration of the connective tissue underlying the epithelium of the gingiva. Skillen[1] has shown that as soon as a crevice forms, regardless of its depth, inflammation occurs in the subepithelial or periodontal tissue. Churchill[2] stated, "Although no clinical symptoms necessarily accompany the deepening of the crevice, we routinely observe in our sections of so-called healthy marginal structures, a small round cell infiltration at the crevice bottom, pointing to a chronic inflammatory reaction." Orban[3] in a clinical and histological study of the gingiva found that the free gingiva is infiltrated even in those specimens which appeared normal clinically. Gallardi[4] regards the presence of lymphocytic infiltration as normal.

The literature dealing in general with the migration of lymphocytes in the epithelium is very extensive. Stöhr,[5] Brieger and Görke,[6] Renn,[7] Hellman,[8] Weidenreich,[9] Hammerschlag,[10] among others studied the penetration of lymphocytes throughout the different layers of the epithelium. This invasion, according to Brieger and Görke,[6] is the consequence of a toxic stimulus. Renn[7] stated that, being a constant occurrence in the epithelium, he considered this invasion as a normal process. According to Jolly[11] a symbiosis exists between the epithelium and the lymphocytes.

The constant presence of lymphocytes in the connective tissue of all the specimens we have studied, and the fact that lymphocytes have been seen by others to migrate into the epithelial layers in various locations, motivated this cytological study of the epithelium of healthy gingivae.

The gingiva is one of the most interesting and important tissues of the oral cavity and gradually is achieving the significance it deserves. The stratified squamous epithelium of the gingiva is well known to histologists. Every textbook describes the layers of the epithelial cells with intercellular bridges and beneath the epithelium, the papillae of the connective tissue. The blood supply to the connective tissue is known, as is the nourishment of the epithelium by the lymph. The gingiva has been the object of a great number of morphological and physiological studies, yet it has not been described as a site of lymphocytic migration. In the oral cavity, the gingiva is constantly under the action of minor trauma and a low degree of irritation related to the presence of bacteria, debris within the sulci around the teeth, and mastication. The condition of the epithelium in the gingival crevice is of prime importance, and as Lundquist[12] stated, "It is significant that the gingival crevices seldom have

an intact epithelial cover. As a result, differences in the reactivity of the epithelium lining the soft tissue side of the crevice seem to determine, in a large measure, the liability to gingival and periodontal involvement. Under the foregoing conditions, the bacteria or their toxic elements constantly seep through to produce low grade inflammatory processes in the underlying connective tissue."

The question of the lymphocytes and of their response to outside stimuli has been raised most frequently during the process of inflammation in which they play such a prominent role. The clinical picture of inflammation has been gradually transformed into a biological concept which considers inflammation as a defense reaction which is more or less uniform throughout the whole animal kingdom.

The lymphocytes have been studied so extensively and the literature is so voluminous that it has seemed inadvisable to attempt a review of the subject. Some of the main aspects will be indicated briefly in connection with our problem. The lymphocytes are found in the blood stream, in lymphatic tissue, in the exudate of the serous cavity, in the red pulp of the spleen, and in the cerebrospinal fluid. Further, the normal loose connective tissue always contains a varying number of ameboid wandering cells.

Among the various authors who have studied the presence of lymphocytes in the epithelium, Schaffer[13] made a very good review and presented a most extensive bibliography. He mentioned the presence of lymphocytes in the epithelium of the esophagus, intestine, trachea, various glands and their ducts, uterus, oviduct, vagina, ureter and bladder, tongue, upper lip, epididymis, cloacal epithelium (in rays) and epidermis. Polak[14] using one of the variations of Del Rio Hortega's stains, has shown in the tonsil the close relationship between lymphocytes and epithelium. More recently, Andrew and his collaborators,[15-20] studied the presence and behavior of the lymphocytes in the intestine, trachea, and epidermis.

There appears to be no mention in the literature, so far as we have found, of the presence of lymphocytes in the epithelium of the gingiva. Considering the widespread occurrence of lymphocytes through the epithelium in various parts of the body, it seems surprising that this matter has not received more attention in the study of the gingiva.

MATERIAL AND METHODS

The present study is based upon the investigation of 50 gingival human biopsy specimens from patients ranging in age from 13 to 55, taken from various gingival regions of both jaws. Biopsies from dogs were also studied. The subjects chosen had no evident pathological condition in the periodontal tissues. In the human material before the biopsies were performed, a careful study of the clinical conditions of the mouth was made and all functional movements of each patient were checked, so as to obtain a more complete picture of the over-all functioning of the teeth. Also full mouth radiographs were taken. The biopsies were taken after measurement of the sulcus. The average depth of the sulci was 2 mm. In the interproximate space a horizontal incision was

made a little beyond the bottom of the sulcus. Care was taken not to traumatize the tissues and immediately after removal, the material was washed and placed in 10 per cent formalin and Zenker-formal solution. After fixation, the tissues were dehydrated, cleared and embedded. Paraffin and e.v. nitrocellulose embedding were used. Labio- and bucco-lingual sections were cut at an average of 6 microns for paraffin and 10 microns for nitrocellulose, and mounted serially. The staining methods used were hematoxylin-eosin, Heidenhain's iron hematoxylin, and that of Maximow, viz., hematoxylin followed by the azure-eosin combination. The sections were studied largely with the immersion lens.

OBSERVATIONS

The general appearance of the lymphocytes before they entered into the epithelium was very similar to that seen in blood or connective tissue in other parts of the body. The shape was more or less spherical and presented the characteristic picture of a thin shell of cytoplasm enclosing a comparatively large nucleus.

Relatively few lymphocytes were found, but their presence was constant in all of the specimens studied. They penetrated the basal cell layer of the epithelium of the gingiva, thus entering into the intercellular spaces. Here they fitted themselves between the epithelial cells. It appears as though these cells were pressed upon by the lymphocytes, and deep impressions in the epithelial cells were made.

In the process of emigration from the lamina propria throughout the basal layer lymphocytes showed decided variations in the shape of their nuclei and cytoplasm, but at times the migration was accomplished with slight changes in cell area and morphology. In their passage through the basal cell layer, cytoplasm and nucleus were drawn out into long irregular shapes. Slender pointed processes were projected between the epithelial cells. Some of the cells showing a small amount of ameboid cytoplasm in front were sometimes so small as to be scarcely visible. They were overshadowed by the epithelial cells. There was usually more cytoplasm than in the normal lymphocyte. The nuclear region was often the broadest part of the cell, but the nucleus itself was very plastic and followed the changes of the cytoplasm in their ameboid movement. The cell assumed an elongated, twisted appearance.

As soon as the lymphocytes adapted themselves between the epithelial cells, they recovered their normal shape. However, there was usually more cytoplasm than for those seen in the connective tissue. The entire body of the cell seemed to increase in size. It appears that the size of lymphocytes is governed by the distribution of nucleus and cytoplasm. The large lymphocytes have relatively more cytoplasm than the smaller cells in which the cytoplasm forms a thin ring around the nucleus. The epithelial cells surrounding the lymphocytes showed no changes in the cytoplasm or in the nucleus, but did show changes in shape due to mechanical pressure of the lymphocytes trying to migrate between them. The distinction between the epithelial cells and the lymphocytes is very marked. The lymphocytes in the basal layer are clearer.

Under resting conditions, the lymphocytes are composed almost entirely of nucleus, and probably the increase in size in the cytoplasm may be due to their having insufficient cytoplasm to send out pseudopodia. In some cases the nuclear membrane was indented, especially towards the surface of the epithelium. There was a predominately basal position of the lymphocytes. A lymphocyte beyond the prickle cell layer of the epithelium was rarely observed.

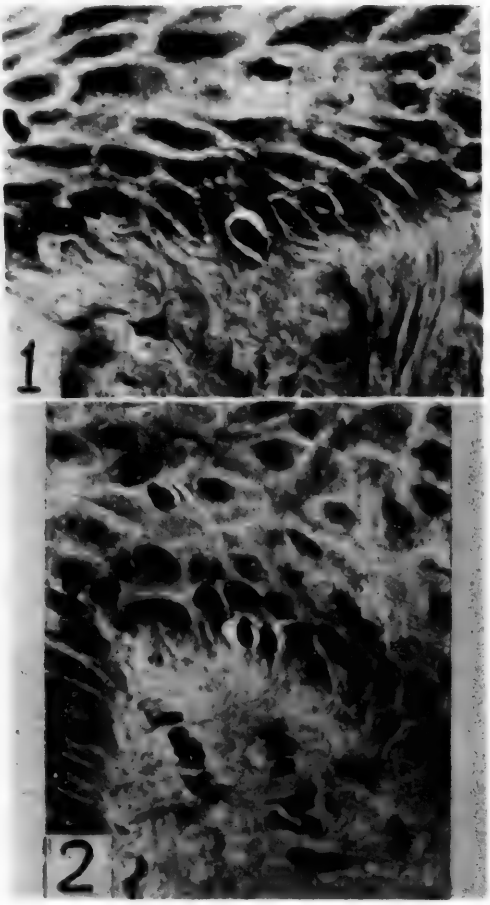

Fig. 1.—Lymphocyte migrating through basal membrane. Half of the body cell is in connective tissue and half in epithelium. The shape of the cell is altered from its normal round to a more flattened shape. The epithelial cells are pressed by the migratory lymphocyte. Harris' hematoxylin-eosin. Orig. mag. ×1210.

Fig. 2.—Lymphocyte in basal membrane layer of epithelium. It shows large clear cytoplasmatic body. Harris' hematoxylin-eosin. Orig. mag. ×1210.

All the lymphocytes were intercellular in position. There was a decided difference between the lymphocytes basally located, especially those passing the first layer of cells in the epithelium, and those that were in the middle of the epithelium. There was a much greater effect on the neighboring cells in most

instances in the basal cell layer. The nucleus very often was surrounded by a very large, clear space or "vacuole" that caused a great deformation of the adjoining epithelial cells, pressing the cytoplasm and the nucleus entirely out of shape. Neither mitoses nor amitoses was observed in the lymphocytes.

DISCUSSION

The physiological meaning of the migration and penetration of the lymphocytes into the epithelium cannot be interpreted as being accidental since this is a general occurrence found in various epithelia. One of the most outstanding

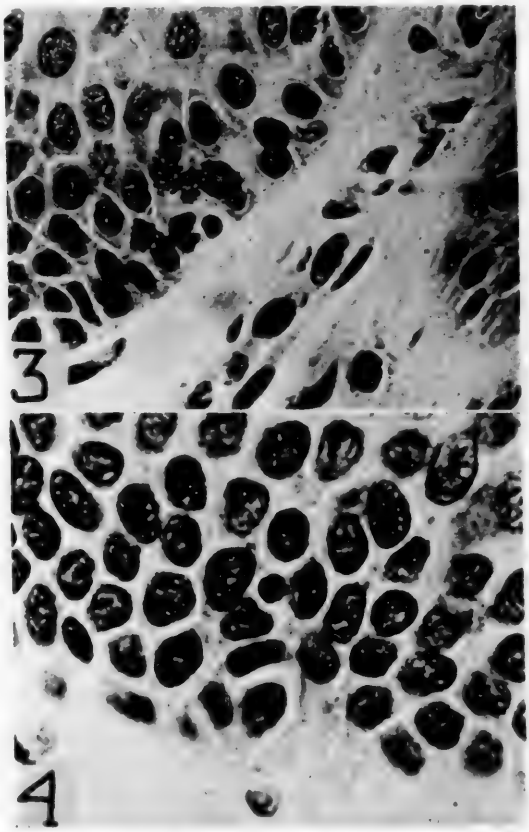

Fig. 3.—Small lymphocyte with large cytoplasmatic body in basal layer of the epithelium. Adjoining epithelial cells have been deformed by the lymphocyte. In right upper angle, a larger lymphocyte with normal cytoplasm is shown in connective tissue. Hematoxylin-eosin. Orig. mag. ×1210.

Fig. 4.—Lymphocyte probably at rest. No change in shape. Intercellular position is clear. Hematoxylin-eosin. Orig. mag. ×1210.

features of this type of cell is its close association in many parts of the body and its interposition between the lumen of various organs and the underlying connective tissue or at the beginning of the lymphatic system in those organs. Its behavior is probably different in different organs, depending upon a specific stimulus or by the influence of the environment or by both.

In interpreting the presence of lymphocytes in the epithelium of the gingiva, the question arises as to the significance of the lymphocytic migration through the epithelium and as to their function.

Mobility was first described in these cells in the amphibians by Ranvier[21] and in the mammals by Arnold,[22] Maximow,[23] Askanazy[24] and has since been confirmed by a great number of investigators.

Lewis and Webster,[25] Sabin,[26] McCutcheon,[27] Henderson[28] among others, have noted that lymphocytes are capable of ameboid movement. Lewis[29] has shown that the mode of the lymphocytic migration is essentially similar to that of the ameba. Ebert, Sanders, and Florey[30] observed in chambers inserted into the rabbit's ear that lymphocytes were very active motile cells. They found that "they are often quite as active as polymorphos, though the most active polymorphos moved more rapidly."

These cells are normally present in the connective tissue and within the blood vessels and lymphatics. In the gingiva the lamina propria carries both blood and lymphatic capillaries close to the epithelial surface, particularly in the papillae that project into the epithelium. From the present study it seems clear that the lymphocyte is a common type of cell found in the epithelium of the healthy gingiva. According to other investigators it possesses ameboid motion and, therefore, it is not unlikely that lymphocytes enter the epithelium by ameboid motion.

Their small number may be accounted for by the fact that under normal conditions the number of cells in the connective tissue is small and, therefore, their neoformation is slight. In this passive resting condition, the developmental potencies of the cells for the most part remain latent and their mutual independence is not manifest.

The normal function of the lymphocyte as a cell is a question which apparently has not been solved. In general, there are two schools of thought concerning the function of the lymphocytes. The first regards the lymphocyte as a multipotential or embryonic cell. One consequence of this view is the acceptance of a unitarian theory of blood histogenesis. The second school denies that the lymphocyte possesses developmental potencies and regards it as a mature and fully differentiated cell. One of the most important problems of lymphocyte behavior thus centers around the question of whether or not the lymphocyte is capable of undergoing transformation into other cell types. Andrew and Andrew[15, 17] describe the possible transformation of lymphocytes into epithelial cells in their studies of the presence and role of the lymphocytes in the normal epidermis. They describe a "clear cell" as a transitional form between the lymphocyte and the epithelial cell, and suggest the possibility of the same role of the lymphocytes in the oviduct, trachea, vagina, tongue and esophagus, whose epithelia show the same type of clear cell and lymphocytes. In our material the "clear cell" type was seen in all the specimens. Further transformation of the lymphocytes was not possible to detect and no lymphocytes were seen in the superficial layer.

The transformation of lymphocytes into other cell types has been shown on sections and on tissue culture. Maximow[31-33] and Bloom[34-37] have demonstrated the development of lymphocytes into polyblasts and fibroblasts and that the monocytes develop by individual transformation of the lymphocytes. Maximow,[23, 38] Weidenreich,[39] Downey and Weidenreich,[40] Bergel,[41] and Jordan,[42] among others, also believe that monocytes develop from lymphocytes.

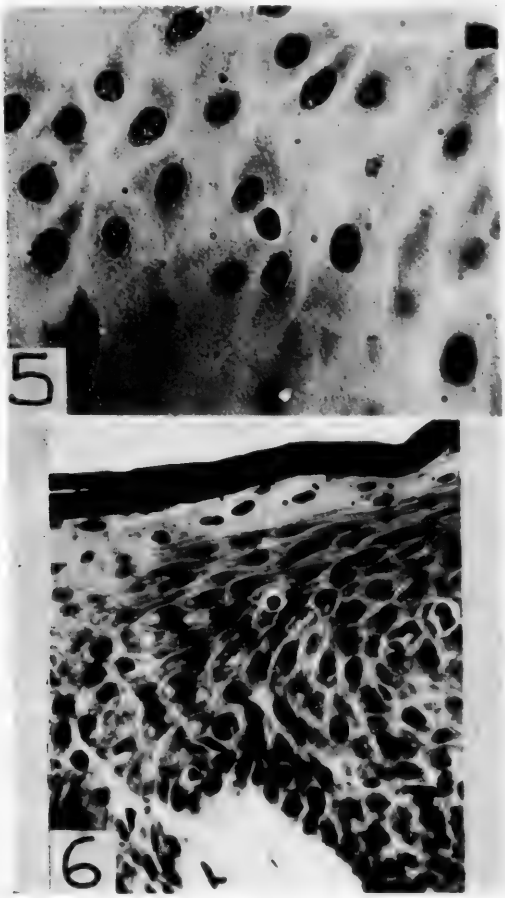

Fig. 5.—Lymphocyte in spinous cell layer. No changes in adjoining epithelial cells. Hematoxylin-eosin. Orig. mag. ×1210.

Fig. 6.—Lymphocyte close to superficial layer. It shows large "vacuole" and indentations in nucleus. Heidenhain's iron hematoxylin. Orig. mag. ×800.

Bloom,[43] and Kingsbury[44] have shown that the small lymphocytes may develop into polymorphonuclear granulocytes. The development of plasma cells from lymphocytes is admitted by practically all investigators, even extreme dualists as Schridde[45] and Naegeli.[46] However, in spite of all the facts already learned in regard to their multipotentiality, in our study we are rather skeptical toward

conclusions as to the lymphocytes being transformed into epithelial cells, considering that we studied fixed material, and we are especially skeptical since such transformations yielded a variety of conflicting views as to the different germ-layer origin of both types of cells.

It has been mentioned that the lymphocytes need a special stimulus in order to develop their migratory capacity. The fact that lymphocytes are found in inflammatory exudates has been taken by Askanazy[47] and others, as evidence that they have been chemotropically attracted. Neither in the literature, nor in experiments has any direct evidence been found that lymphocytes exhibit chemotropism. Although there are many reports in the literature that state to the contrary, Maximow,[48] Clark and Clark,[49] Shade and Mayr,[50] McCutcheon, Wartman, and Dixon,[51] and Dixon and McCutcheon[52] have shown that lymphocytes do not exhibit chemotropism.

In attacking the problem from another angle, it is difficult not to be influenced by the problem of immunity on account of the countless number of bacteria and their toxins in the sulcus.

The concept of inflammation has greatly changed during the last century. Whereas formerly attention was focused on the circulatory phenomena which dominate the clinical picture, the vascular response is now recognized as merely a preliminary reaction designed to deliver certain leucocytes and to stimulate the production of others. The essential phase of inflammation is concerned with the destruction of antigen and the synthesis of antibody. It is a biochemical phenomenon due to the action of enzymes contained in the various leucocytes. There are several papers in the literature describing the presence of enzymes in the lymphocytes but the results are by no means uniform. We are aware of the fact that bacteria are constantly being taken into the system from the alimentary and respiratory tracts. These bacteria, pathogenic or nonpathogenic, are destroyed in the various tissues and organs to which they may be carried by the lymph and blood stream. Normally, the intake of bacteria is relatively slight and the exercise of what are strictly the physiological function of the cells is bringing about the destruction of the same leads, we may be assured of no disturbance.

Under the best conditions there is doubtless a continuous cellular injury in the gingiva, and the presence of lymphocytes may be perhaps merely one form of reparative response to the minor traumatism. Probably by affixing or absorbing toxins, they may contribute to the local immunity to toxins produced by bacteria in the gingival sulcus.

A number of investigations have been reported in recent years on the site of formation of antibodies and the cells concerned with these processes. It was suggested that lymphocytes may have been an important part in the formation of antibodies. In the digestive and respiratory tracts relatively large masses of lymphatic tissue are seen and perhaps they produce antibodies to combat organisms that are present. Perhaps a reason for the presence of lymphatic tissue near the beginning of the digestive and respiratory tracts is that in this position they produce antibodies before the organisms penetrate further into

either tract. This conception of toxin fixation by lymphocytes appears to be supported also by the lymphoid reaction in many pathological cases where it appears that this tissue furnishes both a mechanical and a chemical filtration for the lymph. Marchand[53] stated, "It is very probable that the defense function of the lymph nodes, especially their capacity of reducing the virulence of pathogenic microorganisms is partly due to the activity of the lymphocytes."

Von Ebner[54] showed a photomicrograph of a specimen of a 3½-year-old child in which there was no pocket, and described the lymphocytic infiltration underneath the epithelium as a lymphoid organ. Retterer,[55] and Vincent[56] claimed that the gingiva contain lymphoid follicles ("tonsille gingivale"). Erausquin and Carranza[57] described the lymphocytic infiltration that, according to Siegmund,[58] is the normal lymphocytic reaction of the organs adapted by immunization. According to Haüpl and Lang[59] this is an inflammatory reaction.

All available evidence seems to point to a defense reaction process, and the best explanation of the function of the lymphocytes during the process of passage through the epithelium of the gingiva seems to be that they are in some way "defense" cells. This is in accord with their location and with the function of the lymphoid tissue throughout the body. However, the relationship of the lymphocytes and the epithelial cells, and the possible changes and ultimate fate of such lymphocytes will be a problem for future study. The function of the lymphocytes is still a matter for much investigation and ideas of their function are subject to controversy.

SUMMARY AND CONCLUSION

1. The present study has confirmed the presence of lymphocytes in the epithelium of healthy gingiva.

2. The great majority of lymphocytes are located in the basal cell layer of the epithelium. The prickle cell layer also shows the presence of lymphocytes.

3. Most of the lymphocytes undergo changes in the nucleus and the cytoplasm. These changes consist, in most instances, of increases in amount of the cytoplasm.

4. All the lymphocytes found were intercellular in position.

5. Neither mitotic nor amitotic figures were found in the lymphocytes.

6. Although there is practically no evidence of lymphocytes in either the tissues or blood of the normal organism carrying out any function, there are many observations on the behavior of these cells in inflammation that seem to indicate that the lymphocytes may be "defense" cells.

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APPARATUS FOR EXAMINATION OF THE SO-CALLED ALVEOLO-DENTAL JOINT OF THE RAT

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THE nature of the connection between the teeth and jaws, the so-called alveolo-dental joint, has been poorly investigated because it is difficult to examine. However, it is important because of the possibilities in maxillary orthopedics and partial denture construction offered by better knowledge of this attachment.

An apparatus which enlarges several thousand times has been devised to make visible the movements of the molars. The principle of the method is based on the fact that the displacement of a tooth under strain will produce variations of capacity in a small condensor. These varying frequences are transformed and made visible on the screen of a cathode ray oscillograph (– C.R.O.) and recorded photographically. The apparatus has been used on the denuded jaw bone of the rat.

The apparatus (Fig. 1) is arranged as follows: A small appliance (1) holds the jaw, fixed in any required position, embedded in quick-setting cement. Strain is produced by two arms arrayed horizontally (2). The lower moveable arm applies pressure which may be produced mechanically or electromagnetically, while the upper arm provides the corresponding point. At the end of each arm is a foil, the two forming a condensor. Their distances can be adjusted to 0.5 μ, checked by a micrometer. When the tooth yields to strain the distance between the foils and, therefore, the capacity of the condensor will vary. The condensor is attached to the oscillator (3) by a short insulated cable. From this self-contained oscillator insulated cables lead to the recording instrument (4). The latter will supply rectangular oscillations with amplitudes proportional to the variations in the capacity of the condensor. This alternating current is conducted to the deflecting plates of the Braun electron tube of a C.R.O.* (8) and made visible on the tube screen. Recording is effected by a mirror reflex camera† (9) for single photographs, a photostatic device (10) being connected with the circuit. For taking serial photographs recording camera‡ (11) is used.

A high frequency vibrator (6) supplies impulses which interrupts the picture on the screen by lines at regular intervals. The instrument may be

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*Manufactured by Phillips Company.

†Rolleiflex Camera.

‡Zeiss Ikon.

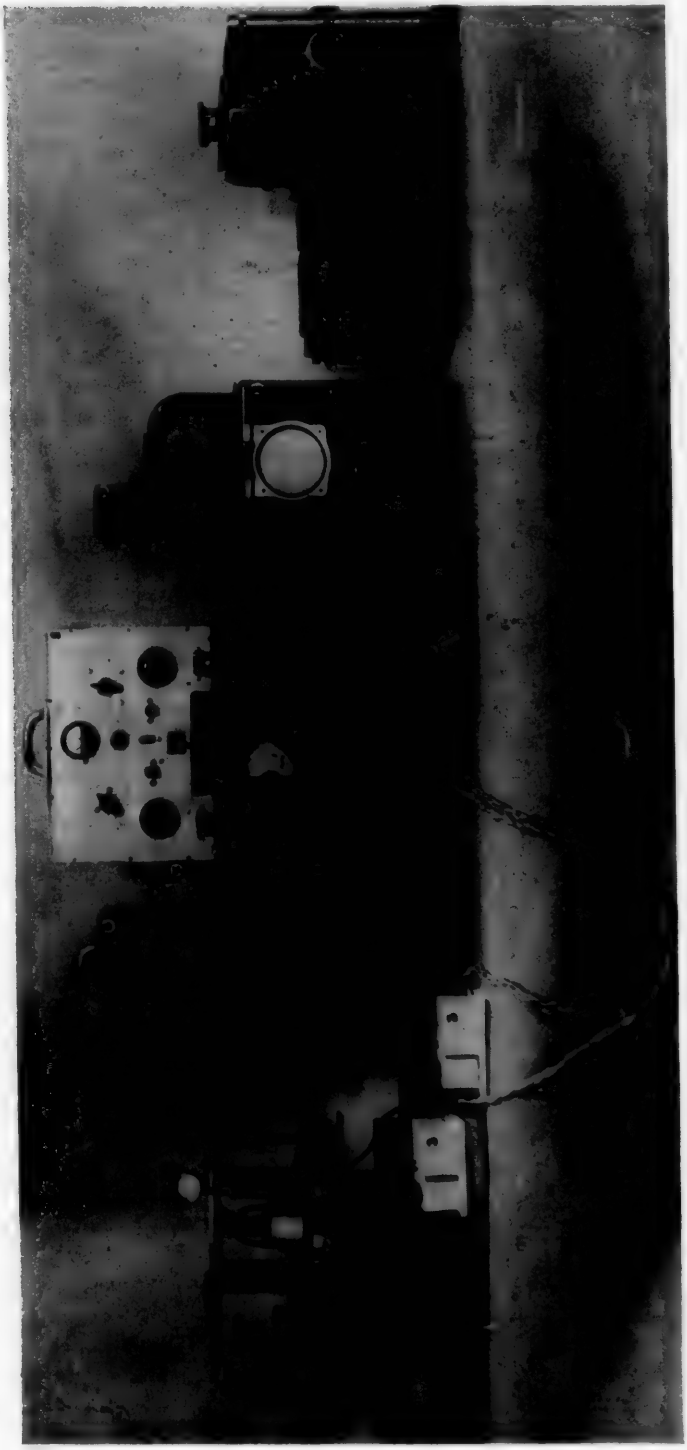

Fig. 1.

checked by a sound generator of 50 cycles (12) controlled by a tuning fork. A switchboard (5) regulates the intensity and duration of the pressure on the tooth being examined.

The following are the advantages of the method: (1) No mechanical forces which might distort the recording process are applied to the condensor and heavy insulation safeguards against external influences on the capacity. (2) The appliance is very sensitive, any deviation from the distance of 0.1 mm. at the condensor plates being amplified 5,000 times on the C.R.O. screen. This can be increased up to 10,000 to 12,000 times. As a result the sequence of the tooth movements may be registered (Fig. 2). (3) As the recording instrument has two oscillators and two oscillator channels it can effect two simultaneous measurements, i.e., the movement of the rat's tooth and of the pressure die may be recorded and compared.

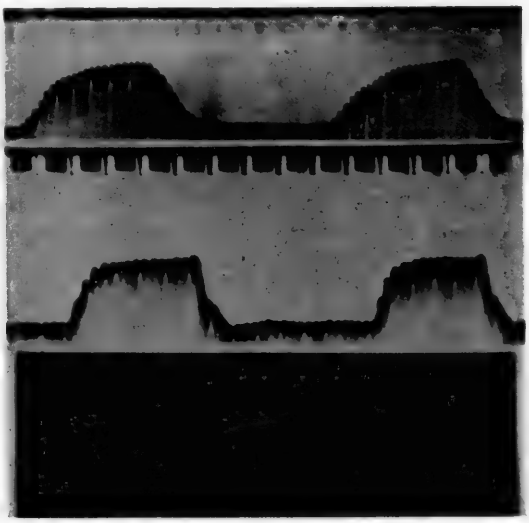

Fig. 2.—Top and middle diagram:—Serial photographs of curves on screen of the C.R.O. obtained by recording camera. Bottom diagram:—Single photograph taken by Rolleiflex. Progress of curves from left to right. Horizontal parts of curves:—teeth at rest. Rising and falling parts of curves:—displacements of teeth. Peaks of curves:—extreme yielding positions of teeth. Right hand slopes of curves:—Movements from resting positions to extreme yielding positions. Left hand slopes of curves:—Return from extreme yielding positions to resting positions. Vertical lines inside curve:—15 Hz timing marks of high frequency vibrator. Black dots in top edge of middle diagram:—50 Hz timing marks of sound generator. Pressure on teeth:—perpendicular to chewing surface, approx. 1.5 kg. by jerks. Ex'ent of greatest displacement:—about 3 μ. Extreme yielding positions reached in about 0.47 seconds. Time taken for return to resting positions:— about 0.12 seconds. Dissimilarity of the three curves due to differencies in biological conditions. General impression:—Tooth movements follow curves of a typical character.

Examples of the graphs obtained are seen in Fig. 2.

The testing apparatus has supplied the following initial results: (1) The rat tooth pressed by jerking movements moves from its resting positions. The movements are not uniform but dissimiliar and of a recurring character. (2) The tooth pressed perpendicularly to its chewing surface (vertically) reaches its extreme position in about 0.47 seconds and returns to resting position in about one-fourth this time. (3) The extreme position from rest is reached

with about 1.5 kg. (4) The rat tooth can be displaced in three dimensions—approximately 3 μ vertically, 15 μ transversely and 1 μ sagitally. (5) When the rat tooth is loaded sagitally the movements are partially absorbed by neighboring teeth. (6) Under pressure the mass not only yields but also tilts about its axes, the number and courses of which remain to be determined.

The results do not yet give a definite picture of the mechanism of the alveolo-dental joint of the rat. Further studies, especially on living animals, are necessary to supply additional information.

SUMMARY

An apparatus has been devised to study the dynamics of the alveolo-dental joint of the rat and preliminary results are reported.

BACTERIAL GROWTH AND CRYSTAL FORMATION

II. Production of Calcium Carbonate Crystals

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THE FORMATION of ammonium magnesium phosphate crystals in association with microbic growth was described in a previous communication.[1] Recently Hewitt,[2] discussing bacterial calculi, reported on the occurrence of calcium carbonate crystals through the metabolic activities of microorganisms. Since information concerning the phenomenon of crystal formation by microorganisms is so meager, a further report on this subject is deemed warranted.

PROCEDURE

The medium suggested by Hewitt[2] was not used because certain constituents contained therein were not at hand. A beef infusion prepared from fresh meat, 1,000 ml.; Difco Bacto-peptone, 10 Gm.; sodium chloride, 5 Gm.; and agar, 20 Gm. was added. Reaction was adjusted to pH 7.2 and 30 ml. phenol red indicator was added. After filtration through cotton the medium was dispensed in 100 ml. amounts and sterilized at 121° C. for 20 minutes. Calcium chloride, 10 per cent solution, Berkefeld filtered, was added to the medium to give a 0.25 to 0.5 per cent concentration. In some lots this addition may cause a lowering of the pH sufficient to inhibit growth, therefore the pH should be readjusted to pH 7.2. A variable amount of precipitate will also form depending primarily on the phosphate, carbonate and protein content of the medium.

Several strains of actinomyces as well as a gram-negative micrococcus and a gram-positive bacillus (a diphtheroid), all isolated from the oral cavity, were used as test microorganisms. They were streaked on the surface of the medium and incubated aerobically. The rapidity with which calcium carbonate crystals occurred is dependent on the species of the microorganisms and their metabolic activities. The micrococcus and the diphtheroid produced crystals in a few days while one strain of actinomyces took more than one week and the other strain produced no crystals. The crystals of $CaCO_3$ are found in greater numbers on top of the colonies and in lesser numbers in the medium in the immediate vicinity of the peripheries of the colonies. At first the crystals are likely to be rather small and observable only by magnification (40×) (Figs. 1 and 2). As the crystals aggregate into larger masses they are noticeable with lower magnification.

The addition of too much calcium chloride to the medium will produce calcium carbonate crystals which are not necessarily the result of bacterial metabolism. The mere pressure of the loop will mechanically abrade the surface of the medium, changing the surface tension and influence crystal deposition; therefore, each plate of medium should be controlled by streaking a portion of

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the agar with a sterile loop. If crystals appear along such sterile streak lines this is an indication that the amount of calcium chloride added to the medium was too large and must be reduced. It must be remembered that the protein,

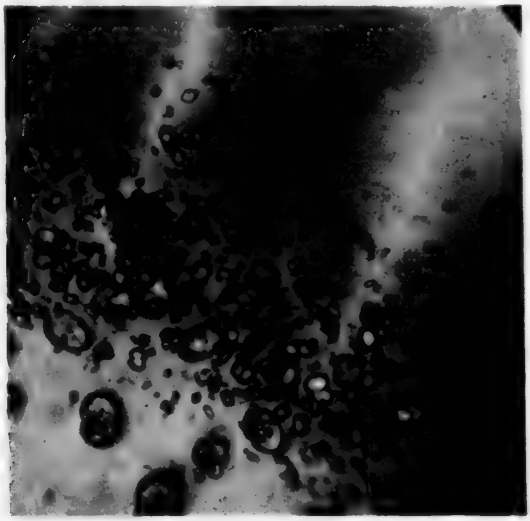

Fig. 1.—Calcium carbonate crystals, two-week-old actinomyces culture, (40×).

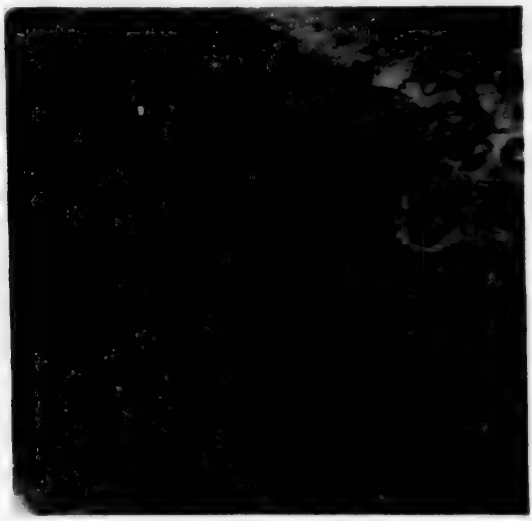

Fig. 2.—Calcium carbonate crystals, gram-negative micrococcus colony, one-week-old culture, (40×).

phosphate and carbonate content of meat infusion agars vary with (1) the source and age of the meat, and (2) the method employed in preparation of the infusion and the medium. Variation in the components of the peptone and the

agar added to the infusion must also be considered. While Hewitt was able to employ calcium chloride in concentration as high as one per cent in some lots of his medium, I found that over half this amount tended to give false results with my infusion agars. While the mere presence of a microbic colony on the surface of a medium must cause some change in surface tension, this factor does not seem to play a major role in crystal formation inasmuch as the colonies of only some species of microorganisms are associated with this phenomenon. Neither the moistness nor dryness of colonial growth could be ascertained as contributing to crystal formation. Hewitt states that only the colonies of proteolytic microorganisms are the instigators of calcium carbonate crystal production.

The addition of dextrose to the medium may enhance the growth of the microorganisms. If fermentation occurs, the acid produced will change the color of the phenol red indicator in the medium to yellow. No crystals are found in an acid environment.

Drying does not appear to be a factor since plates of medium kept 1 month, some uninoculated and others inoculated with microorganisms not associated with crystal formation, showed no evidence of crystal deposition.

DISCUSSION

Hewitt[2] reported that a part of the calcium added to the medium is bound to protein material while the remainder is precipitated in the form of phosphates and carbonates. Bacteria in the course of their nitrogen metabolism, he claimed, decompose the protein bound portion with liberation of ammonium carbonate which combines with calcium ions to form crystals of calcium carbonate. Hewitt also discussed the probable relationship of this process to the production of salivary calculus and stones in the salivary ducts as well as in other regions. The formation of ammonium magnesium phosphate crystals previously reported entails a somewhat similar process wherein the action of microbic enzymes on proteins liberates ammonia which combines with the phosphate and magnesium ions present in the medium. Thus we have 2 in vitro examples of crystal formation through bacterial metabolism. Naeslund[3] presented the theory that salivary calculus was formed as a result of an increase in local alkalinity together with a diminution in protective protein colloid through the enzyme activity of actinomyces which caused a precipitation of calcium salts from the saliva. Similar microbic metabolic activity in vitro induces crystal formation affording further corroboration of Naeslund's theory.

SUMMARY

The formation of calcium carbonate crystals as a product of bacterial metabolism is recorded. The in vitro formation of crystals is of interest as a supplemental fact in the further corroboration of the Naeslund theory of salivary calculus formation.

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THE EFFECTIVENESS OF AN AMMONIUM ION TOOTHPOWDER IN THE CONTROL OF DENTAL CARIES

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THE findings of Kesel, O'Donnell, Kirch and Wach[1] and of Kesel[2] indicated that an ammonium ion dentifrice might be effective in reducing caries activity. Publication of these preliminary findings stimulated a widespread interest in dentifrices containing 5 per cent dibasic ammonium phosphate and 3 per cent urea. Certain commercial enterprises prepared ammonium ion dentifrices for public sale and this in turn contributed to the increasing public interest in the use of this dentifrice as a caries control measure.

We were aware of the extensive study being conducted at Aurora and Peoria by Kerr and Kesel.[3] In that study one group of children brushed their teeth with an ammonium ion dentifrice, another group with a nonammoniated dentifrice. For both these groups toothbrushing was carried out in schools under strict supervision.

Our study was designed to determine the effectiveness of an ammonium ion dentifrice in a group of children and young adults when the actual brushing procedure was not under rigid supervision. That is, we sought to determine whether or not a commercial dentifrice would reduce caries activity in actual practice. The manufacturers of this toothpowder readily agreed to the project and financed the undertaking.

SELECTION OF MATERIAL

High school children in the age group of 12 to 18 years and young adults in the age group of 19 to 31 years were selected for study. Of the 401 participants in March, 1950, 339 remained cooperative throughout the study period, March to November, 1950.

Participants from the following sub-groups volunteered to take part in the project: sub-group 1, final year dental students; sub-group 2, girls from boarding school *A*; sub-group 3, girls from boarding school *B*; sub-group 4, boys from boarding school *C*; sub-group 5, boys from boarding school *D*; sub-group 6, first year teacher's training college students; sub-group 7, boys and girls living at home and attending day schools.

Participants in each sub-group were allotted to either the experimental or the control group by random selection. The composition of the experimental and control groups is outlined in Table I.

METHOD

Examination.—All participants were given a careful clinical and radiographic examination at the beginning of the academic year (March, 1950) and at the end of the academic year (November, 1950). Examinations were made with mouth mirrors and sharp probes and explorers. Compressed air was used to dry each tooth surface before it was examined.

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Recording.—Clinical findings were called to a nurse who recorded them on the participant's chart. This helped to ensure that the examiner was unaware of the group to which each participant belonged.

Radiographic Interpretation.—The clinical findings as recorded were then amended following a careful interpretation of bite-wing radiographs. In an endeavour to standardise radiographic interpretation, all radiographs were examined by one of us (G. N. D.). A viewing box and a magnifying glass were used for this purpose.

TABLE I
COMPOSITION OF GROUPS AND SUB-GROUPS

SUB-GROUP	NUMBER	AGE RANGE (YEARS)	NUMBER IN EXPERIMENTAL GROUP	NUMBER IN CONTROL GROUP
1	26	21-31	12	14
2	29	12-18	15	14
3	48	12-18	25	23
4	32	12-18	19	13
5	48	12-18	24	24
6	71	18-27	32	39
7	85	12-18	44	41
Total	339	12-31	171	168

Susceptibility Tests.—All participants were given two sterile sample bottles and two pieces of paraffin at the time of examination. Each participant was instructed to collect a sample of saliva before getting out of bed on two successive mornings. The time of collection was standardised at 5 minutes. Each sample of saliva was then submitted to a lactobacillus count, and a Snyder's test. Participants from sub-groups 3, 4 and 5 submitted three series of samples: (a) at the beginning of the study period; (b) one month after the beginning of the experiment; and (c) at the end of the study period. Participants from sub-groups 1, 2, 6, and 7 submitted two series of samples: (a) at the beginning of the study period and, (b) at the end of the study period.

Distribution of Toothpowder.—Each tin, whether it contained control or experimental powder, was labelled with the name of the person who was to use the powder which it contained, and with the manufacturers directions, namely: "Brush teeth upon arising and before retiring or as directed by your dentist. Pour ½ teaspoonful of powder on side of a clean glass. Dip wet toothbrush in powder and brush teeth. Pour water on remaining powder in glass (until ⅓ full) and rinse mouth with solution." Powder was distributed immediately following the initial examination and new supplies were distributed when the previous powder was consumed. Participants were given no information which would enable them to determine whether the powder they were using was control or experimental. All toothpowder was prepared by Bristol Myers Pty. Ltd. The experimental powder was compounded according to the formula of, and on license to, the University of Illinois Foundation. The control powder contained the same ingredients as the experimental powder, except that it was tinted pink and it did not contain the active principles (5 per cent dibasic ammonium phosphate and 3 per cent urea). In this study, therefore, the control powder was similar to toothpowder "A" in Kerr and Kesel's experiment.[3] The experimental powder was the same as toothpowder "B" of Kerr and Kesel.[3]

RESULTS

Effect on the Annual Increment of Dental Caries.—In the control group of 168, the total number of tooth surfaces which became carious during the study period was 1081; a mean increase of 6.43 new carious tooth surfaces per person. In the experimental group of 171, the total number of tooth surfaces which became carious during the study period was 1099; a mean increase of 6.43 new carious tooth surfaces per person.

The frequency distribution of tooth surfaces which became carious during the period of study is shown in Table II.

TABLE II
FREQUENCY DISTRIBUTION OF TOOTH SURFACES WHICH BECAME CARIOUS DURING THE PERIOD OF STUDY

NEW CARIOUS TOOTH SURFACES	0-2	3-5	6-8	9-11	12-14	15-17	18-20	21-23	24-26	TOTALS
Control group	39	47	35	17	15	10	3	1	1	168
Experimental group	31	47	50	21	9	7	3	2	1	171
Totals	70	94	85	38	24	17	6	3	2	339

Although these results show no difference between the experimental and control groups it was thought desirable to examine the data more closely with a view to determining the effects of the following variables: (1) sub-group, (2) number of times per day that the powder was used, (3) the age of the participants, and (4) the sex of the participants.

Analysis of differences in sub-groups.—Data on the mean increase in D.F. tooth surfaces per person in each of the seven sub-groups are given in Table III.

TABLE III
ANALYSIS OF RESULTS IN TERMS OF DIFFERENCES IN SUB-GROUPS

SUB-GROUP	GROUP	NUMBER	TOTAL INCREASE IN D.F. TOOTH SURFACES	MEAN INCREASE IN D.F. TOOTH SURFACES PER PERSON
1	Experimental	12	45	3.75
	Control	14	68	4.85
2	Experimental	15	103	6.86
	Control	14	113	8.07
3	Experimental	25	151	6.04
	Control	23	116	5.05
4	Experimental	19	144	7.63
	Control	13	85	6.53
5	Experimental	24	148	6.17
	Control	24	144	6.00
6	Experimental	32	245	7.66
	Control	39	238	6.10
7	Experimental	44	263	5.98
	Control	41	317	7.70

If the differences in these sub-groups are expressed in terms of percentage differences the apparent results in the experimental group are a reduction of

22.6 per cent in sub-group 1, 14.9 per cent in sub-group 2, and 22.3 per cent in sub-group 7. However, an analysis of these findings, the results of which appear in Table IV, indicates that these differences are not statistically significant. On the other hand, four sub-groups (3, 4, 5 and 6) showed a higher increase in the experimental group than in the control group. The apparent result is an increase of 19.6 per cent in sub-group 3, 16.9 per cent in sub-group 4, 2.8 per cent in sub-group 5, and 25.5 per cent in sub-group 6. Again these differences are not statistically significant.

TABLE IV

STATISTICAL ANALYSIS OF RESULTS FOR SUB-GROUPS 1, 2, AND 7

SUB-GROUP	GROUP	MEAN INCREASE IN DF SURFACES PER PERSON	(I) ΣD^2	DIFF. OF MEANS	STD. ERROR OF DIFF. OF MEANS*	(II) 'T'	(III) 'η'	'P'
1	Exptl.	3.75	52.66	1.10	1.51	0.72	24	.50
	Control	4.85	307.68					
2	Exptl.	6.86	91.74	1.21	1.41	0.86	27	.50
	Control	8.07	301.26					
7	Exptl.	5.98	750.98	1.72	1.06	1.62	83	.10
	Control	7.70	1240.49					

*Calculation of Standard error of difference of means:

Mean increase in DF surfaces in experimental group = 5.98
Mean increase in DF surfaces in control group = 7.70
Difference of means = 1.72

$$\text{Combined estimate of variance, } S^2 = \frac{750.98 + 1240.49}{43 + 40} = 23.99$$

$$S = \sqrt{23.99} = 4.8985$$

$$\text{Standard error of difference of means} = 4.8985 \sqrt{\frac{1}{44} + \frac{1}{41}} = 1.062$$

(i) = Sum of the deviations squared.
(ii) = Ratio of difference of means to the standard error of difference of means.
(iii) = Degrees of freedom.

Analysis of differences in terms of the number of times per day that the toothpowders were used: From the results obtained, the details of which are given in Table V, it is apparent that there is no significant difference between experimental and control groups when the toothpowders were used one, two, three or four times per day.

TABLE V

STATISTICAL ANALYSIS OF RESULTS IN TERMS OF THE NUMBER OF TIMES PER DAY THAT THE TOOTHPOWDERS WERE USED

NUMBER OF TIMES PER DAY	GROUP	NUMBER	MEAN INCREASE IN DF SURFACES PER PERSON	ΣD^2	DIFF. OF MEANS	STD. ERROR OF DIFF. OF MEANS	'T'	'η'	'P'
1	Experimental	23	6.34	414.84	2.79	1.495	1.86	44	0.1
	Control	23	9.13	716.58					
2	Experimental	115	6.56	2324.25	0.36	0.639	0.56	118	0.5
	Control	105	6.20	2570.00					
3	Experimental	19	4.21	105.08	1.18	1.131	1.03	35	0.25
	Control	18	5.39	309.17					
4	Experimental	6	8.17	386.82	1.17	4.659	0.25	9	0.5
	Control	5	7.00	156.00					

Analysis of differences with age: Details of the results under this heading are given in Table VI. From these data it would appear that in the largest

TABLE VI
ANALYSIS OF RESULTS IN TERMS OF AGE (LAST BIRTHDAY) IN YEARS

AGE LAST BIRTHDAY IN YEARS	CONTROL GROUP			EXPERIMENTAL GROUP			DIFFERENCE OF MEANS
	NUMBER	TOTAL INCREASE IN D.F SURFACES	MEAN INCREASE IN D.F SURFACES PER PERSON	NUMBER	TOTAL INCREASE IN D.F SURFACES	MEAN INCREASE IN D.F SURFACES PER PERSON	
12	10	58	5.80	19	136	7.15	+1.35
13	30	264	8.80	27	173	6.40	−2.40
14	28	151	5.39	30	166	5.53	+0.14
15	25	169	6.76	22	152	6.90	+0.14
16	15	91	6.06	17	115	6.76	+0.70
17	22	126	5.72	17	112	6.58	+0.86
18	12	62	5.16	18	143	7.94	+2.78
19	6	53	8.83	6	35	5.83	−3.00
20	3	22	7.33	1	7	7.00	−0.33
21	1	6	6.00	1	3	3.00	−3.00
22	1	9	9.00	5	14	2.80	−6.20
23	4	13	3.25	3	25	8.33	+5.08
24	5	22	4.40	1	6	6.00	+1.60
25	2	14	7.00	3	10	3.33	−3.67
26	2	6	3.00	0	-	-	-
27	1	8	8.00	0	-	-	-
28	1	7	7.00	0	-	-	-
31	0	-	-	1	2	2.00	-
Totals	168	1081	6.43	171	1099	6.43	0.00

group, the 13 year olds, a beneficial effect has resulted. An analysis of this age group gave the following result:

Mean increase in D.F tooth surfaces in the experimental group	=	6.40
Mean increase in D.F tooth surfaces in the control group	=	8.80
Difference of the means	=	2.40
Standard error of difference of means	=	1.392
'T'	=	1.724
Degrees of freedom	=	55
$0.1>P>0.05$		

From this analysis it is apparent that there is no significant difference between the experimental and control groups of the 13 year olds. The same is true for all other age groups.

Analysis of differences in sex: Details of the results under this heading appear in Table VII. There is no significant difference between the experimental and control groups for either males or females.

TABLE VII
ANALYSIS OF RESULTS IN TERMS OF DIFFERENCES IN SEX

SEX	CONTROL GROUP			EXPERIMENTAL GROUP			DIFF. OF MEANS
	NUMBER	TOTAL INCREASE IN D.F SURFACES	MEAN INCREASE IN D.F SURFACES PER PERSON	NUMBER	TOTAL INCREASE IN D.F SURFACES	MEAN INCREASE IN D.F SURFACES PER PERSON	
Females	85	551	6.48	85	557	6.55	+0.07
Males	83	530	6.38	86	542	6.30	−0.08
Totals	168	1081	6.43	171	1099	6.43	0.00

Effect on the Acidogenic Bacteria of the Mouth.—

Frequency distribution of Lactobacillus counts in the experimental and control groups: Lactobacillus counts were graded as follows:

No Lactobacilli per ml. of saliva ---------- = (−)
1-40,000 Lactobacilli per ml. of saliva ------ = (+)
40,000-500,000 Lactobacilli per ml. of saliva = (++)
Over 500,000 Lactobacilli per ml. of saliva -- = (+++)

The frequency distribution of these counts in each group at the beginning of the experiment is given in Table VIII. A Chi-Square Test was applied to these data with the following result:

TABLE VIII

DISTRIBUTION OF LACTOBACILLUS COUNTS AT THE BEGINNING OF THE EXPERIMENT

GROUP	LACTOBACILLUS COUNT				TOTALS
	−	+	++	+++	
Experimental	18	25	65	52	160
Control	12	19	76	40	147
Totals	30	44	141	92	307

$\chi^2 = 3.898$
Degrees of freedom = 3
$0.30 > P > 0.20$

This indicates that there was no significant difference between the experimental and control groups at the beginning of the study.

The frequency distribution of Lactobacillus counts in each group at the end of the experiment appears in Table IX. A Chi-Square Test was applied to these data with the following result:

TABLE IX

DISTRIBUTION OF LACTOBACILLUS COUNTS AT THE END OF THE EXPERIMENT

GROUP	LACTOBACILLUS COUNT				TOTALS
	−	+	++	+++	
Experimental	20	30	82	28	160
Control	20	27	72	28	147
Totals	40	57	154	56	307

$\chi^2 = 0.236$
Degrees of freedom = 3
$0.98 > P > 0.95$

This indicates that there was no significant difference between the Experimental and Control Groups at the end of the experiment.

Changes in individual Lactobacillus counts during the course of the experiment:

Sub-groups 3, 4, and 5: In these sub-groups Lactobacillus counts and Snyder's tests were taken at the beginning of the study period, one month after the beginning of the experiment, and at the end of the study period. The number and percentage of participants showing, (a) increased counts and increased

Snyder test results, (b) decreased counts and decreased Snyder test results, and (c) no change in counts or Snyder test results, at the end of one month are given in Table X.

TABLE X

CHANGE IN SUSCEPTIBILITY TESTS ONE MONTH AFTER BEGINNING OF THE EXPERIMENT (SUB-GROUPS 3, 4, AND 5)

	LACTOBACILLUS COUNT						SNYDER'S TEST					
	INCREASE		DECREASE		NO CHANGE		INCREASE		DECREASE		NO CHANGE	
GROUP	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE
Experimental	13	25	23	44	16	31	5	10	26	50	21	40
Control	16	31	9	18	26	51	8	16	18	35	25	49

Chi-Square Test was applied to the Lactobacillus data with the following result:

$$\chi^2 = 8.729$$
$$\text{Degrees of freedom} = 2$$
$$0.02 > P > 0.01$$

From this result it would seem that a significant change has occurred in the Lactobacillus counts of the experimental group at the end of one month's use of the toothpowder.

The Susceptibility Test results at the end of the experiment are compared with the results at the beginning of the experiment in Table XI. The Chi-

TABLE XI

CHANGE IN SUSCEPTIBILITY TESTS AT THE END OF THE EXPERIMENT (SUB-GROUPS 3, 4 AND 5)

	LACTOBACILLUS COUNT						SNYDER'S TEST					
	INCREASE		DECREASE		NO CHANGE		INCREASE		DECREASE		NO CHANGE	
GROUP	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE	NO.	PERCENTAGE
Experimental	12	18	23	35	31	47	31	47	12	18	39	60
Control	16	26	17	28	28	46	6	11	26	47	24	42

Square test was applied to the Lactobacillus data in this table with the following result:

$$\chi^2 = 1.419$$
$$\text{Degrees of freedom} = 2$$
$$0.50 > P > 0.30$$

This result would appear to indicate that the significant change in the Lactobacillus counts of the Experimental Group, which took place after the toothpowder had been used for one month, was not maintained throughout the study period.

All sub-groups combined: The Susceptibility Test results at the end of the experiment are compared with the results at the beginning of the experiment in Table XII. The Chi-Square Test was applied to the Lactobacillus data in this

TABLE XII

CHANGE IN SUSCEPTIBILITY TESTS AT THE END OF THE EXPERIMENT
(ALL SUB-GROUPS COMBINED)

	LACTOBACILLUS COUNT						SNYDER'S TEST					
	INCREASE		DECREASE		NO CHANGE		INCREASE		DECREASE		NO CHANGE	
GROUP	NO.	PER-CENT-AGE	NO.	PER-CENT-AGE	NO.	PER-CENT-AGE	NO.	PER-CENT-AGE	NO.	PER-CENT-AGE	NO.	PER-CENT-AGE
Experimental	35	22	57	35	69	43	38	24	76	47	47	29
Control	31	21	54	37	62	42	28	18	69	45	55	36

table with the following result:

$$\chi^2 = 0.081$$
$$\text{Degrees of freedom} = 2$$
$$0.98 > P > 0.95$$

This indicates that no significant change has occurred in the Lactobacillus counts of the Experimental Group after the use of the experimental toothpowder for one academic year.

DISCUSSION

In this experiment there was no controlled supervision of actual toothbrushing by the participants but all cans of toothpowder, whether control or experimental, had attached to them exactly the same directions as appear on the cans of the commercial ammonium ion toothpowder under test.

In defense of the ammonium ion toothpowder it could be argued that perhaps, despite assurances, all participants did not use the powder as directed. This argument may have some substance.

Since this study was concluded the results of Kerr and Kesel's[3] experiment have been published. In the latter experiment, which was designed to determine the effect of both supervised oral hygiene and an ammoniated dentifrice, this factor was under excellent control. Children in a group using a nonammoniated dentifrice, Dentifrice *A* and those using an ammoniated dentifrice, Dentifrice *B*, brushed their teeth under supervision in schools.

If from Kerr and Kesel's data we wish to determine whether or not an ammoniated dentifrice is more effective than a nonammoniated dentifrice when used under similar conditions, the real control group for the group using the ammoniated dentifrice is the group using Dentifrice *A* and not the unsupervised control group that they have taken. In our experiment, which was designed to determine whether or not an ammonium-ion toothpowder was more effective than a nonammoniated toothpowder in actual practice, the control powder was similar to Dentifrice *A* in Kerr and Kesel's experiment, and the experimental powder was exactly the same as Dentifrice *B*.

Kerr and Kesel found that the percentage of teeth attacked in a two-year period was less in the group using an ammoniated dentifrice than in the group using a nonammoniated dentifrice under the same controlled conditions. When actual brushing procedure is not supervised we found no difference between these two groups over a period of one academic year.

A statistical analysis of Kerr and Kesel's data gives interesting results. In Table XIII the results from Kerr and Kesel's Control group who had no super-

TABLE XIII

ANALYSIS OF DATA FROM KERR AND KESEL'S EXPERIMENT
CONTROL AND DENTIFRICE *A* GROUPS

	TEETH ATTACKED 1948-1950	TEETH NOT ATTACKED 1948-1950	TOTAL TEETH (UNATTACKED 1948)
Controls (no brushing in school)	492	3351	3843 (n_1)
Dentifrice *A* (Nonammoniated)	304	2310	2614 (n_2)
Totals	796	5661	6457

Statistical analysis:

Percentage of control teeth attacked, p_1 = 492/3843 = 12.80 per cent
Percentage of control teeth unattacked, q_1, = 3351/3843 = 87.20 per cent
Percentage of Dentifrice *A* teeth attacked, p_2 = 304/2614 = 11.62 per cent
Percentage of Dentifrice *A* teeth unattacked, q_2 = 2310/2614 = 88.38 per cent
Percentage difference between two groups = $p_1 - p_2$ = 1.18 per cent

Standard error of difference between two proportions $= \sqrt{\frac{p_1 \times q_1}{n_1} + \frac{p_2 \times q_2}{n_2}}$

$$= \sqrt{\frac{12.80 \times 87.20}{3843} + \frac{11.62 \times 88.38}{2614}}$$

$= 0.825$

Ratio of observed difference to its standard error $= 1.18/0.825$
$= 1.43$

That is, the difference between the control and Dentifrice *A* groups is not significant.

vised brushing in school, and the group who used a non-ammoniated dentifrice under supervision, are analyzed. This analysis reveals that the difference between these two groups could have occurred by chance. In other words, there is no significant difference between a group using any dentifrice at home and a group using a non-ammoniated powder under supervision in school. In Table XIV the results from a group using a nonammoniated dentifrice and a group

TABLE XIV

ANALYSIS OF DATA FROM KERR AND KESEL'S EXPERIMENT
DENTIFRICE *A* AND DENTIFRICE *B* GROUPS

	TEETH ATTACKED (1948-1950)	TEETH NOT ATTACKED (1948-1950)	TOTAL TEETH UNATTACKED (1948)
Dentifrice *A* (Nonammoniated)	304	2310	2614 (n_2)
Dentifrice *B* (Ammoniated without bentonite)	254	2240	2494 (n_3)
Totals	558	4550	5018

Statistical analysis:

Percentage of Dentifrice *A* teeth attacked, p_2 = 304/2614 = 11.62 per cent
Percentage of Dentifrice *A* teeth unattacked, q_2, = 2310/2614 = 88.38 per cent
Percentage of Dentifrice *B* teeth attacked, p_3, = 254/2494 = 10.18 per cent
Percentage of Dentifrice *B* teeth unattacked, q_3, = 2240/2494 = 89.82 per cent
Percentage difference between two groups, $(p_2 - p_3)$ = 1.44 per cent

Standard error of difference between two proportions $= \sqrt{\frac{p_2 \times q_2}{n_2} + \frac{p_3 \times q_3}{n_3}}$

$$= \sqrt{\frac{11.62 \times 88.38}{2614} + \frac{10.18 \times 89.82}{2494}}$$

$= 0.87$

Ratio of observed difference to its standard error $= 1.44/0.87$
$= 1.65$

That is, the difference between the Dentifrice *A* and Dentifrice *B* groups is not statistically significant.

using an ammoniated dentifrice under similar conditions, are analyzed. This analysis indicates that the differences between these two groups could have occurred by chance.

In view of these findings it is interesting to note, as shown in Table XV, that a highly significant difference is obtained when a group using any dentifrice under no control is compared with a group using an ammoniated dentifrice under strictly controlled conditions of brushing.

TABLE XV

ANALYSIS OF DATA FROM KERR AND KESEL'S EXPERIMENT
CONTROL AND DENTIFRICE *B* GROUPS

	TEETH ATTACKED (1948-1950)	TEETH NOT ATTACKED (1948-1950)	TOTAL TEETH UNATTACKED (1948)
Controls (no brushing in school)	492	3351	3843 (n_1)
Dentifrice *B* (Ammoniated without bentonite)	254	2240	2494 (n_2)
Totals	746	5591	6337

Statistical analysis:

Percentage of control teeth attacked, p_1 = 492/3843 = 12.80 per cent
Percentage of control teeth unattacked, q_1, = 3351/3843 = 87.20 per cent
Percentage of Dentifrice *B* teeth attacked, p_2 = 254/2494 = 10.18 per cent
Percentage of Dentifrice *B* teeth unattacked, q_2 = 2240/2494 = 89.82 per cent
Percen age difference between two groups ($p_1 - p_2$) = 2.62 per cent

Standard error of difference between two proportions $= \sqrt{\frac{p_1 \times q_1}{n_1} + \frac{p_2 \times q_2}{n_2}}$

$$= \sqrt{\frac{12.80 \times 87.20}{3843} + \frac{10.18 \times 89.82}{2494}}$$

$$= 0.87$$

Ratio of observed difference to its standard error = 2.62/0.87
= 3.01

That is, the difference between the control and Dentifrice *B* groups is highly significant.

It is apparent from these findings and our own results that the evidence in favour of an ammonium ion dentifrice must be viewed with some circumspection. From neither study can we conclude that the unsupervised use of an ammonium ion dentifrice is any more effective than any nonammoniated dentifrice.

SUMMARY AND CONCLUSIONS

1. An ammonium ion dentifrice was tested for its effectiveness in controlling dental caries under conditions which simulate the use of the ordinary commercial product.

2. Under the conditions of the test the experimental ammonium ion toothpowder failed to reduce the annual increment of dental caries when its effect was compared with that of a control toothpowder.

3. In no sub-group was there a significant difference between the control and experimental groups.

4. The number of times per day that the powder was used made no apparent difference to this result.

5. No significant difference due to sex or age was manifest either in the control or experimental groups.

6. In three sub-groups the experimental powder produced a significant change in Lactobacillus counts after this powder had been used for one month.

7. In the same three sub-groups this significant change was not manifest at the end of the experiment.

8. At the end of the study period there was no significant difference between the Lactobacillus Counts of the control and experimental groups.

9. The results of Kerr and Kesel's study are analyzed and discussed.

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INFLUENCE OF AMMONIA AND OF UREA UPON L. ACIDOPHILUS 4646

II. Studies on Anaerobic Glycolysis

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IN CONNECTION with their investigation of methods for controlling dental caries, Kesel, O'Donnell, Kirch, and Wach,[1-5] reported that dibasic ammonium phosphate was inhibitory toward the growth of *L. acidophilus* 4646 (recently reclassified as *L. casei*), in vitro. It was stated by these investigators that the toxic effect of ammonium phosphate could be demonstrated when the salt was present in relatively low concentration, approximately 0.08 Molar, provided that the reaction of the growth medium was slightly alkaline. Evidence was presented to show that urea enhanced the potency of the effects. They further stated that the formation of acids in incubating mixtures of saliva and glucose was inhibited by ammonium phosphate and by urea.

It was been demonstrated,[6] that the inhibitory effect of these compounds upon growth of pure cultures of lactobacilli is critically sensitive to variations in the salt composition and the hydrogen ion concentration of the growth media. Other investigators have reported failure of ammonium ions to inhibit growth of oral lactobacilli, although these reports did not specify whether alkaline pH was maintained in the medium throughout the course of incubation.[7]

Stephan,[8] and Muntz and Miller[9] found that exposure of natural and "artificial" dental plaques to strong solutions of urea prevented the rapid fall in pH and the formation of lactic acid which otherwise ensued upon subsequent exposure to glucose.

In the present paper, evidence is presented which indicates that, in the case of suspensions prepared from pure cultures of *L. acidophilus* 4646, ammonium ions do not exert a specific inhibitory effect upon acid formation from glucose but that glycolytic activity of these suspensions is depressed by urea in high concentrations.

METHODS

Anaerobic glycolysis can be measured conveniently under controlled conditions by means of the Warburg respirometer. When the reactions are carried out in bicarbonate buffer equilibrated with CO_2, a constant pH is maintained at constant temperature and stoichiometric amounts of CO_2 are liberated, equivalent

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to the acid produced. The resulting change in gas pressure is measured. This technique lends itself to the measurement of extremely small changes in the quantity of the metabolites concerned.*

L. acidophilus 4646 was grown in broth similar to that described by Tsuchiya and Halvorson,[10] consisting of peptone 5 Gm., yeast extract 10 Gm., glucose 5 Gm., tap water 100 ml. and distilled water 900 ml., adjusted with NaOH to pH 7.0 and autoclaved for 15 minutes under 15 pounds pressure. After a 20-hour incubation at 37° C. the bacteria were harvested by centrifugation. The yield was usually small, about 0.5 ml. of packed cells per liter of medium, but the suspensions glycolyzed rapidly. The organisms were washed twice with cold 0.5 per cent stock salt mixture.† They were then resuspended and diluted in 4 per cent stock salt mixture to make 1 or 2 per cent suspensions by volume, as desired. In the packed condition they could be kept frozen at –30° C. for at least 3 months without appreciable loss of activity.

Double side-arm Warburg vessels were employed. The final concentrations of material in the vessels were: glucose, 0.01 M; $NaHCO_3$, 0.02 M‡; salt mixture and cell suspension, 0.5 ml.; temperature, 37.9 ±0.05° C.; gas, 5% CO_2-95% N_2; pH, ca. 7.4; the total fluid volume was 2.0 ml.

The glycolytic activity was expressed as microliters of carbon dioxide evolved per hour per 0.1 mg. cell-nitrogen ($Q_{\text{lactic acid}} = \mu\, 1{\cdot}CO_2/hr/0.1$ mg. N).

The nitrogen determinations were performed in triplicate upon samples of the 2 per cent cell suspension before it was added to the reaction vessels. To 1 ml. of cell suspension in a Kjeldahl flask was added 1 ml. of 50 per cent H_2SO_4. The mixture was digested for 30 minutes, 2 drops of 30 per cent hydrogen peroxide added, and digestion was continued for another 15 minutes. The flask was allowed to cool and was then connected directly to an improvised Pregl tube. Two milliliters of H_2O and 3 ml. of 40 per cent NaOH were run into the flask and the ammonia was steam-distilled for 3 minutes into 10 ml. of 2 per cent boric acid containing 2 drops of a mixture of 0.1 per cent methyl red and 0.1 per cent bromeresol green. The boric acid was titrated back to the endpoint with 0.020 N H_2SO_4 from a Koch microburette, and the nitrogen values were calculated. Digested ammonium sulfate standards containing 1 mg. N could be recovered consistently with a precision of 100 ±0.3 per cent. The cell-nitrogen found in samples of the various "2 per cent" cell suspensions was usually in the neighborhood of 0.5 mg. N. per milliliter.

Ammonium phosphate and potassium phosphate buffers, pH 7.4, were prepared by mixing appropriate amounts of equimolar solutions of the monobasic and dibasic forms of each salt.

*For complete description of the Warburg techniques see Umbreit, W. W., Burris, A. H., and Stauffer, J. F. Manometric techniques. Minneapolis, Burgess, c1945. 203 pp.

†Salt mixture, stock solution:—

	%
$MgSO_4 \cdot 7H_2O$	4.0
NaCl	0.2
$MnSO_4 \cdot H_2O$	0.2
$FeSO_4 \cdot 7H_2O$	0.2

The $FeSO_4$ was prepared separately and a small quantity of HCl was added to retard oxidation of the iron during storage.

‡One volume of washed packed cells suspended in 50 volumes of wash solution containing 2 volumes of salt mixture.

In the experiments all reactions were run in duplicate or triplicate vessels. The cells, salts, and buffers were placed in the main body of the flasks, and glucose, water, or test solution was added from the side-arms after gassing and temperature equilibration were completed. The usual thermobarometer was used to control variations due to changes in atmospheric pressure during the experiments.

Manometer readings were made at 10 minute intervals. In the presence of substrate, glycolysis began immediately and proceeded at a constant rate for 1 to 2 hours, depending upon the concentration and activity of the cells and the rate of exhaustion of the substrate.

Preliminary experiments indicated that the rate of CO_2 evolution was approximately the same from preparations at pH 7.4 and at pH 6.1.

EXPERIMENTAL

Effect of Ammonium Phosphate on Glycolytic Activity.—To study the effects of ammonia on glycolysis under slightly alkaline conditions, the reactions were run at pH 7.4 and ammonium phosphate buffer was added in serially increasing concentrations. Equimolar potassium phosphate buffer was substituted in vessels which were run parallel to these, and which were otherwise identical. Endogenous activity was observed in vessels in which distilled water was substituted for glucose. (For results see Table I and Fig. 1.)

It was apparent that the glycolytic activity of the suspensions was depressed progressively in the presence of increasing concentrations of phosphate. The ammonium salt was not especially more effective than the potassium salt, and in the lowest concentration, 0.0072 M, ammonium phosphate appeared to exert a mild stimulatory effect. This stimulation, and the apparent higher activity of all of the ammonia-containing suspensions, may be a reflection of the stimulating effect of NH^+_4 upon glycolysis and transphosphorylation, an effect which is considerably greater than that of K^+.[11, 12]

TABLE I. EFFECT OF AMMONIUM AND POTASSIUM PHOSPHATES ON GLYCOLYTIC ACTIVITY OF L. ACIDOPHILUS 4646 IN BICARBONATE BUFFER, pH 7.4, AT 38° C

FLASK-CONCENTRATION OF ADDED BUFFER (MOLARITY)	GLYCOLYTIC ACTIVITY μL. CO_2/HR./0.1 MG. CELL-NITROGEN AVERAGES OF TRIPLICATES	
	POTASSIUM PHOSPHATE	AMMONIUM PHOSPHATE
0.00	98.0	98.0
0.0072	92.3	115.8
0.027	83.0	92.0
0.05	69.7	86.6
0.10	46.8	55.8
0.15	29.1	38.9
0.20	22.8	26.5
0.38	7.4	13.2

Effect of Urea on Glycolytic Activity.—To observe the influence of urea upon the glycolytic activity of intact washed lactobacilli, the organisms were grown and prepared as described. The experimental conditions were substantially the same as those employed in the previous section.

After gas and temperature equilibration, glucose was added to the cells from a side-arm and carbon dioxide evolution was followed for 40 minutes. Urea was then added from the other side-arm to give final concentrations of 2 M, 3 M, and 4 M, and the activity was followed for 70 minutes longer. Each urea-containing vessel was controlled with a comparable vessel from which the glucose substrate had been omitted.

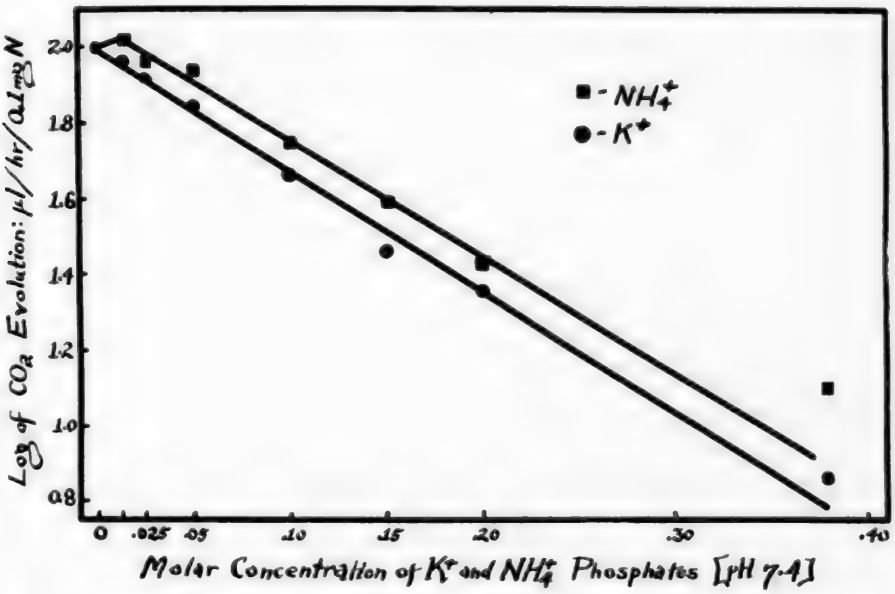

Fig. 1.—Effect of phosphate upon glycolytic activity of *L. acidophilus* 4646, measured by carbon dioxide evolution from bicarbonate buffer, pH 7.4, at 38° C.

The rate of glycolysis was comparable in all of the vessels before the addition of urea (Fig. 2). After the urea was added, a momentary decrease in pressure occurred, which incidentally was not due to fluctuation in pH. During the following 10-minute interval, a new equilibrium was reached, and the rate of glycolysis was observed to have declined in proportion to the quantities of urea which were present. Inhibition was almost complete in the presence of 4 M urea, but not in the presence of 3 M or 2 M urea.

Effect of Exposure to Urea Prior to Glycolysis.—Muntz and Miller[9] showed that glycolysis in dental plaque material was almost completely inhibited after exposure to 8 M urea for 10 minutes. Since in the preceding experiments almost complete inhibition was obtained with only 4 M urea, it was desired to determine whether previous exposure to this concentration would lower the future glycolytic activity of the lactobacilli.

Ten milliliters of a 2 per cent suspension (by volume) of routinely washed cells were dispensed into each of 4 tubes, and the contents were centrifuged for 15 minutes. The supernatant fluids were carefully aspirated and discarded. The cells in Tube 1 were resuspended in 25 ml. of 0.5 per cent salt mixture;

those in Tube 2, in 25 ml. of 4 M sodium chloride; and those in Tube 3, in 25 ml. of 4 M urea. After mixing, these tubes were incubated at 37° C. for one hour. After the first 30 minutes had elapsed, 25 ml. of 4 M urea were added to the cells in Tube 4, and this suspension was incubated for only 30 minutes.

At the end of the incubation period, the suspensions were centrifuged for 15 minutes at room temperature, and the supernatant fluids were aspirated as completely as possible. The tubes were inverted for 5 minutes to drain them well. The supernatant fluids were tested with a Macbeth pH meter and were found to have remained unchanged at pH 7.0.

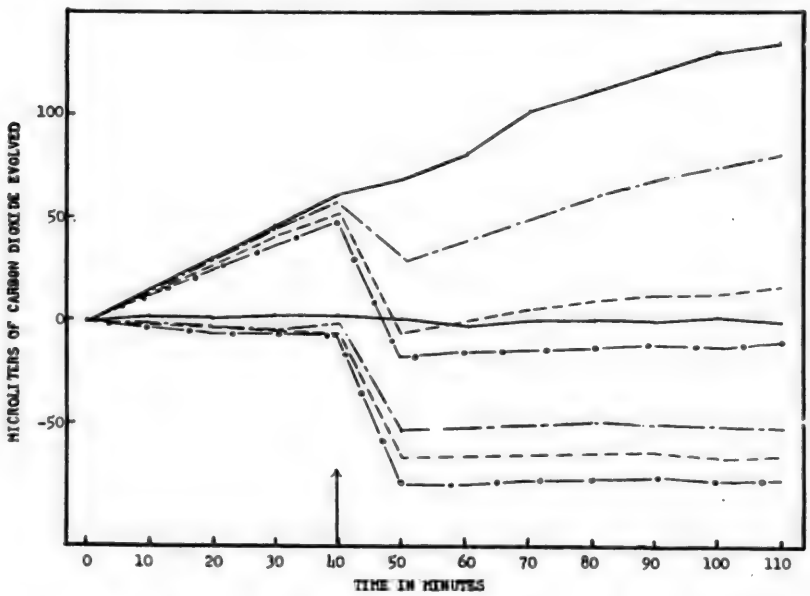

Fig. 2.—The effect of added urea on the glycolytic activity of *L. acidophilus* 4646 in bicarbonate buffer, pH 7.4, at 38° C. ——— No urea; •—•—• 2 M urea; — — — 3 M urea; o—o—o 4 M urea. Lower curves represent endogenous activity. Urea was added after 40 minutes.

The packed cells were then resuspended in 70 ml. of cold 0.5 per cent salt mixture and were recentrifuged and decanted. To each tube was then added 20 ml. of 2 per cent salt mixture, to obtain a 1 per cent cell suspension. The residual urea and NaCl were thus diluted out to at least 1/1400 of their original concentrations. Aliquots were removed from each tube for dry cell weight determinations.

The Warburg flask conditions were essentially the same as in the preceding experiments, except that a mixed sodium-potassium phosphate buffer (0.075 M, pH 7.4) was included in all vessels. The glucose solutions and the water controls were held in the side-arms, and contained no buffer, so that a small decrease in pressure was observed immediately after the side-arms were emptied.

For each of the 4 cell suspensions a glucose-free control was run along with the glycolyzing preparations. Activity was followed for 80 minutes. The findings are shown in Table II and Fig. 3.

TABLE II. EFFECT OF PRIOR EXPOSURE TO UREA UPON GLYCOLYTIC ACTIVITY OF L. ACIDOPHILUS 4646 IN BICARBONATE BUFFER, pH 7.4

TUBE NO.	TREATMENT	DURATION OF PREINCUBATION	DRY WEIGHT OF CELLS PER VESSEL	NET CO_2 EVOLVED PER HOUR	PER CENT INHIBITION
1	0.5% salts	1 hour	2.09 mg.	80 µl.	—
2	4 M NaCl	1 hour	2.04 mg.	53 µl.	34
3	4 M Urea	1 hour	2.05 mg.	32 µl.	60
4	4 M Urea	½ hour	2.03 mg.	33 µl.	60

It is seen that the activity of the cells which had been preincubated with 4 M urea was much lower than that of cells preincubated with the salt mixture, and that the effects were not significantly strengthened by preincubation for longer than 30 minutes.

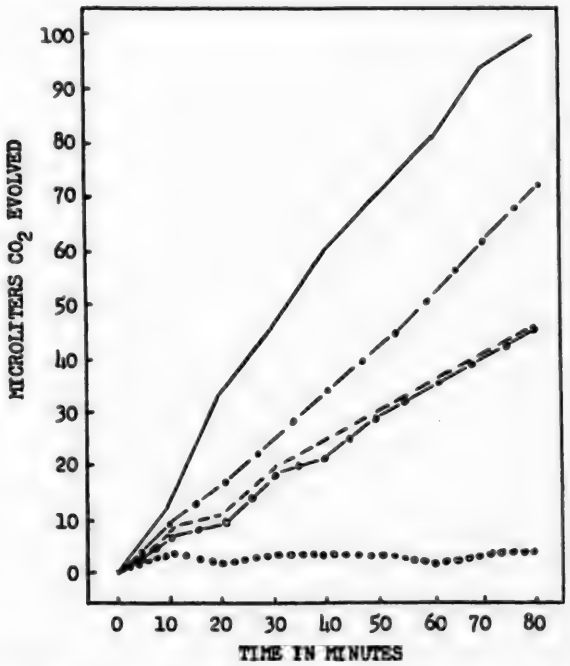

Fig. 3.—Glycolytic activity of *L. acidophilus* 4646, in bicarbonate buffer, pH 7.4, at 38° C after preincubation with various solutions. (a) —— Pre-incubated with salt mixture (60 minutes); (b) •—•—• Pre-incubated with 4 M NaCl (60 minutes); (c) — — — Pre-incubated with 4 M Urea (60 minutes); (d) o—o—o Pre-incubated with 4 M Urea (30 minutes); (e) ooooo Endogenous activity of preparations without glucose.

The partial inhibition obtained in the case of the 4 M NaCl "control" may be attributed to a "salting-out" effect upon proteins, or to some specific toxic property of the sodium or chloride ions. Utter[13] has demonstrated that glycolyzing rat brain homogenates are inhibited by sodium ions as a result of the stimulatory effect of these ions upon an apyrase present in his preparations. It is possible that a similar mechanism may have produced the effect observed in these experiments, although the concentration of residual sodium was much lower than that employed by Utter.

Absence of Urease Activity in L. acidophilus 4646.—Because ammonium salts in high concentration are capable of depressing the activity of the cells, it was important to determine whether the added urea had been hydrolyzed to ammonium carbonate during the course of the foregoing experiments. It was unlikely that spontaneous hydrolysis had occurred under the conditions employed, and although Stephan stated that lactobacilli exhibit no urease activity,[14] this feature was investigated with suspensions of the cells.

A modification of the method of Conway[15] was employed to estimate ammonia. In the modification, Warburg vessels were used in place of the usual Conway dishes. Standard quantities of ammonia could be recovered consistently with good internal agreement in proportionality. With flask conditions as described, except that a 3.3 per cent cell suspension (9.15 mg. dry weight) was present in each vessel, there was no detectable ammonia formation from 0.017 M urea in 3 hours with or without glucose. Ammonia standards were recovered completely from control vessels.

DISCUSSION

These studies indicate that while *L. acidophilus* 4646 may not flourish in the presence of 5 per cent ammonium phosphate under certain conditions,[6] nonproliferating cells may continue to produce acid from glucose.

The mechanism of the inhibition observed with both ammonium and potassium phosphates was not investigated further. It may be speculatively attributed to electrostatic or osmotic effects, or to the inactivation of magnesium or other cofactors of the glycolytic enzymes by the formation of phosphate complexes.

A 0.38 M solution of dibasic ammonium phosphate is a 5 per cent solution of that salt. This is 5 times the final concentration used by Kesel O'Donnell, Kirch, and Wach in their growth experiments. On the basis of the present experimental findings, it was considered that the nonspecific depressant effect manifested by ammonium phosphate upon the glycolytic activity of washed cell preparations of lactobacilli would not adequately account for the inhibition of growth nor for the prevention of acid formation in saliva-glucose mixtures as reported by those investigators.

Urea was effectively inhibitory toward glycolysis only when its concentration approached 4 Molar (24 per cent). The maintenance of this level in the mouth is impractical, but short exposures are reported to be effective[8] in retarding acid production in the dental plaque.

The mechanism by which urea affects the activity of the organisms has not been elucidated. The absence of urease activity indicated that the influence of urea upon the glycolytic activity of the organisms in preceding experiments could not be ascribed to either the alkalinity or toxicity of formed ammonia. However, there exists a possibility that urea may enter into the metabolic processes of the cells in a manner by which no ammonia is liberated into the medium. This matter could be investigated further using isotopic urea.

In related experiments[16] it was found that urea inhibited acid formation in saliva-glucose mixtures, but the effective urea concentrations invariably caused

coagulable material in the saliva to precipitate. Furthermore, in a number of studies on bacterial respiration in whole saliva, in which lactobacilli play a very small part, oxygen consumption, too, was inhibited by strong solutions of urea. The observations strongly suggest that the inhibitory action of urea is closely associated with its ability to denature many types of protein, as mentioned by Muntz and Miller,[9] and that its behaviour toward lactobacilli is probably nonspecific. It should be borne in mind that the oral fluid abounds with many acidogenic streptococci and other forms, whose susceptibility to ammonia and urea have not been demonstrated.

It may be implied that the introduction of ammonium phosphate and of urea into the oral cavity should not be expected to cause immediate inhibition of acid formation, unless the concentration of these compounds is maintained at a high level, and the reaction of the fluid is kept alkaline.*

SUMMARY AND CONCLUSIONS

1. A study has been made of certain effects of ammonium phosphate and of urea upon the glycolytic activity of nonproliferating cell suspension of *L. acidophilus* 4646.

2. The rate of glycolysis with intact cells at pH 7.4 was depressed in the presence of either ammonium phosphate or potassium phosphate buffers, in proportion to the concentration of the buffers. NH_4^+ and K^+ exhibited no significant difference in inhibitory power under the conditions employed.

3. In the presence of 4 M urea, the glycolytic activity of washed-cell preparations was almost completely inhibited. Lower concentrations of urea were less effective.

4. Preincubation of washed lactobacilli in 4 M urea for 30 minutes resulted in marked reduction of their glycolytic activity thereafter. The effect was not heightened by longer preincubation.

5. No appreciable urease activity was detected in suspensions of these organisms.

6. The implications of the findings are discussed briefly.

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*Since the submission of this and the preceding paper,[6] other important reports have appeared. Kerr and Kesel[17] have presented the results of a well-controlled, large scale, clinical investigation of the effects of an ammoniated urea dentifrice upon dental caries in school children. Jenkins and Wright[18, 19] have reported upon their extensive study of ammonia and urea in relation to acid production in saliva and to the growth and acid-forming characteristics of *L. acidophilus* 4646. Certain of their experimental findings and conclusions are substantiated or confirmed in the present communications.

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FREQUENCY OF ORAL MICROORGANISMS THAT INFLUENCE LACTOBACILLUS GROWTH IN VITRO

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IN AN earlier publication[1] a review of the literature indicated that the idea of possible bacterial antagonism among the oral flora had been recorded a number of years previously, but the experimental data illustrating the antagonism are limited. With the use of the agar-streak-plate test the investigators in this laboratory had found that a random sample of the bacteria from pooled saliva indicated this antibacterial activity was more prevalent than previously described. However, the streak-plate method and pooled saliva did not lend itself to any concrete estimation as to the frequency of this phenomenon in individual salivas and the relative abundance of these bacteria in measured quantities of saliva. The investigation reported here gives some information in this connection.

MATERIALS AND METHODS

Saliva samples were collected from 98 young adult individuals. Immediately upon arising, the donor chewed sterile paraffin for three minutes and deposited all saliva in a sterile bottle. The sample was brought to the laboratory by 8:00 A.M. and immediately refrigerated until 1:00 P.M. when it was used.

To determine the bacterial antagonistic activity, a modification of one of the techniques described by Waksman[2] was used.

The saliva sample was shaken 3 minutes in a Kahn shaker. One c.c. of the saliva was then placed in 9 c.c. of sterile saline. The 1:10 dilution was further diluted in saline to 1:100, 1:1000, and 1:10,000. The 1:10 dilution was discarded, since preliminary work indicated it would not yield isolated colonies upon plating. One-tenth c.c. portions of the 1:100, 1:1000, and 1:10,000 dilutions were placed in 10 c.c. of melted blood agar base (42° C.), thoroughly agitated by hand and poured into Petri dishes. These plates were incubated 72 hours at 37° C. At the end of this incubation period, the growth on these plates was covered with a thin film of tomato agar which had been previously inoculated with a heavy suspension of lactobacilli. The tomato juice agar was prepared by adding 0.5 c.c. of a 48 hour growth of Hadley lactobacillus (A. T. C. 4646) in 2 per cent sucrose broth to 10 c.c. of melted (42° C.) pH 6.1 tomato juice agar. The plates were reincubated 96 hours at 37° C.

At the end of the second incubation period the plates were examined on a Quebec colony counter. Over and around the saliva bacterial colonies, in some instances the lactobacillus showed no growth, in other instances an area of lactobacillus stimulation or satellitism was observed.

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Blood agar base, yeast dextrose agar and proteose peptone broth were used in establishing pure cultures of the organisms producing these two phenomena. Since the stimulatory organisms grew rather easily upon subculturing, a random sample of fourteen different colony types were isolated. Subcultures of seventy-five individual inhibition colonies from twenty-five salivas were attempted, but only eleven survived.

Using blood agar base media the pure cultures were tested for antagonistic action by the agar streak-plate method. The test organism pure culture was streaked across the plate and incubated 72 hours at 37° C. Then each of three strains of lactobacilli were cross streaked into the test organism at right angles. The three lactobacilli were: A. T. C., H-4646,* and one isolated from saliva. The plates were then reincubated for 96 hours at 37° C., after which the distance from the test organism showing no lactobacillus growth was noted.

The agar well technique[3] with blood agar base media was used to test the pure cultures for saliva compatability. Pour plates using the test organism as the inoculum were made 2-3 mm. deep and wells 10 mm. in diameter were cut. Freshly collected salivas were placed in the wells and the plates incubated 72 hours at 37° C. At this time the plates were examined for a growth-free zone around the wells. Each pure culture was tested against sixteen different salivas.

RESULTS

The double pour-plate technique showed that 90 of the 98 salivas examined contained bacteria that influenced the growth of the Hadley lactobacillus. Eighty-nine of the salivas had colonies that produced areas of larger lactobacillus colonies—stimulation or satellitism (Fig. 1). Twenty-five salivas had colonies that produced zones free of lactobacillus growth—inhibition (Fig. 2). In some instances the same saliva colony exhibited both phenomena (Fig. 3). All the salivas which showed inhibition also showed some stimulation with one exception. Hence only eight salivas failed to demonstrate both phenomena.

The number of these organisms per c.c. of saliva is indicated in Table I. The number for the stimulatory activity ranged from zero to 3,700,000, with most salivas below one million. Most of the salivas with the inhibitory activity gave counts of ten to twenty thousand.

The cross-streak test results of the pure cultures are given in Table II. It can be seen that most of the inhibitory organisms surviving pure culture isolation still showed the same phenomenon. However, the original stimulatory organisms also demonstrated the inhibition.

The types of organisms established in pure culture are given in Tables III and IV. It can be seen that a wide variety of forms were found. The attempt to secure some idea concerning the growth compatibility of the organisms with saliva also is given in Tables III and IV. Good compatability was evidenced by most of the organisms with almost all the salivas used. Neither did good nor poor compatibility seem to be especially linked with any special type of organism.

*A lyophilized strain secured from the Regional Agriculture Laboratory at Peoria, Ill.

Fig. 1. Fig. 2.

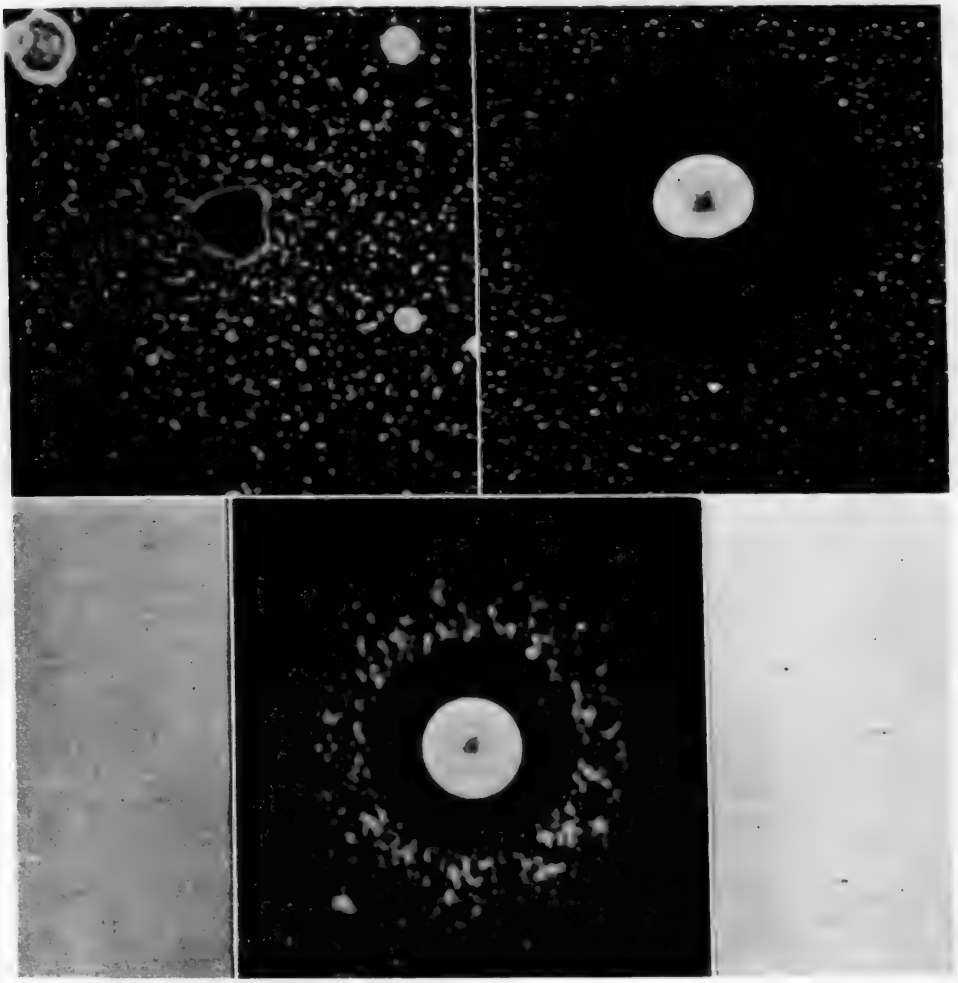

Fig. 3.

Fig. 1.—Saliva bacterial colony showing an area of lactobacillus growth stimulation.
Fig. 2.—Saliva bacterial colony showing an area of lactobacillus growth inhibition.
Fig. 3.—Saliva bacterial colony showing an area of lactobacillus growth inhibition and an area of stimulation.

DISCUSSION AND SUMMARY

The results of this investigation would seem to indicate that the saliva from most individuals contains appreciable numbers of bacteria that can have an influence on lactobacillus growth, but it is recognized that too much significance should not be placed on any exact numerical evaluation. This is not unique to this bacterial count technique; however, the pour-plate technique does indicate the inhibition and stimulation occurrence is of sufficient magnitude to offer a supply of source material for investigation of these phenomena. Several interesting relationships of these phenomena already are apparent.

The difference between the good stimulatory and poor inhibitory pure culture survival is striking, but not understood. Whether a relatively large group of the inhibitory organisms represent anaerobic forms, or require some specific vitamin or some other special culture procedure for continued growth remains to be determined.

TABLE I

NUMBER ORGANISMS PER C.C. SALIVA SHOWING STIMULATORY AND INHIBITORY ACTIVITY TO HADLEY LACTOBACILLUS

	NO. INDIVIDUALS	ORGANISMS PER C.C. SALIVA
STIMULATION	9	
	14	1-100,000
	9	101-200,000
	12	201-300,000
	13	301-400,000
	8	401-500,000
	7	501-600,000
	4	601-700,000
	3	701-800,000
	1	801-900,000
	3	901-1,000,000
	9	1,001-1,500,000
	5	1,501-2,000,000
	1	3,700,000
INHIBITION	1	200
	1	1,000
	1	3,000
	13	10,000
	5	20,000
	1	40,000
	1	50,000
	1	70,000
	1	90,000

TABLE II

RESULTS OF CROSS-STREAK TEST OF ORGANISMS ORIGINALLY SHOWING STIMULATORY AND INHIBITORY ACTIVITY WITH DOUBLE POUR-PLATE TECHNIQUE

	NO GROWTH OF TEST ORGANISM	NO INHIBITION	INHIBITION	TOTAL
Original inhibition	2	2	7	11
Original stimulation	0	2 (after 24 hours)	12	14
Total	2	4	19	25

Tested with 3 strains of Lactobacilli.

The relationship, if any, of the stimulation and inhibition is not clear. They could represent two different phenomena, but the pour-plate technique demonstrated both with the same bacterial colony (Fig. 3). In addition, the cross-streak test showed as good inhibition with original stimulation colonies as with original inhibition colonies. Additional evidence is needed before a conclusion can be reached.

Since the definitive oral flora is not known and apparently is difficult to determine, the status of these organisms in this connection likewise is not settled. All that can be said is that the well tests seem to indicate most of them can grow when saliva exerts some influence on their environment. It is recognized also that the nature of the substances causing both the stimulation and inhibition has not been determined.

It is hoped continued investigation will provide additional information concerning these phenomena.

TABLE III

WELL TECHNIQUE SALIVA COMPATABILITY OF ORGANISMS ORIGINALLY SHOWING STIMULATION OF HADLEY LACTOBACILLUS

	ORGANISM	NUMBER OF SALIVAS			
		NO INHIBITION	RETARD	INHIBITION	TOTAL
R_1	Mycobacteriaceae or Actinomycetaceae	*10	2	4	16
R_2	Micrococcus epidermidis	*16	0	0	16
R_3	Micrococcus candidus	*16	0	0	16
R_4	Micrococcus candidus	*15	(1, no growth)		16
R_5	Sarcina flava	*15	1	0	16
R_6	Lactobacillus sp.	*growth too poor to evaluate*			
R_7	Phycomycetes sp.	*16	0	0	16
R_8	Nocardia sp.	2	7	7	16
R_9	Streptococcus lactis or durans	*14	0	2	16
R_{10}	Bacillus subtilis	9	0	7	16
R_{11}	Micrococcus epidermidis or Graffkya tetragena	*16	0	0	16
R_{12}	Mycobacteriaceae or Actinomycetaceae	(1, no growth) 3	6	6	16
R_{13}	Bacillus brevis	*16	0	0	16
R_{14}	Lactobacillus sp.	*growth too poor to evaluate*			

*Good Compatability	9
Poor Compatability	3
No evaluation	2
Total	14

TABLE IV

WELL TECHNIQUE SALIVA COMPATABILITY OF ORGANISMS ORIGINALLY SHOWING INHIBITION OF HADLEY LACTOBACILLUS

	ORGANISM	NUMBER OF SALIVAS			
		NO INHIBITION	RETARD	INHIBITION	TOTAL
H_1	Micrococcus luteus	*14	0	2	16
H_2	Corynebacterium xerase	*12	1	3	16
H_3	Micrococcus luteus or urease	*16	0	0	16
H_4	Mycobacteriaceae or Actinomycetaceae	*growth too poor to evaluate*			
H_5	Corynebacterium xerase	5	8	2	15
H_6	Mycobacteriaceae or Actinomycetaceae	*growth too poor to evaluate*			
H_7	Bacillus brevis	*16	0	0	16
H_8	Bacillus cereus	*15	1	0	16
H_9	Micrococcus epidermidis	*16	0	0	16
H_{10}	Bacillus brevis	*16	0	0	16
H_{11}	Gaffkya tetragena	*growth too poor to evaluate*			

*Good Compatability	7
Poor Compatability	1
No evaluation	3
Total	11

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THE EVANSTON DENTAL CARIES STUDY

VII. The Effect of Artificially Fluoridated Water on Dental Caries Experience of 12-, 13-, and 14-Year-Old School Children

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FORMER reports from The Evanston Dental Caries Study have been concerned with the significance of artificially fluoridated drinking water and its influence on dental caries experience. Briefly these reports are as follows:

(1) The determination of fluorine in teeth, in dental cements and in foods purchased locally; (2) the outline of the study, the school clinics, and clinical and radiographic procedures; (3) the large numbers of communal water supplies containing fluorides found in Indiana, Ohio, Illinois, South Dakota, and Texas and the number of people having access to these waters grouped according to fluorine strengths; (4) the most prevalent type of lactobacillus, using the Hadley and Bunting classification. Also after 12 months of fluorine there was a displacement of about 5 per cent of the cases of the 6- and 8-year-old children from the higher count groups (over 1000 lacto per milliliter) to the lower count groups (those under 1000 ml.); (5) the relationship of the fluorine content of saliva on the prevalence of dental caries and that of the fluorine content of saliva on the lactobacillus counts; (6) the caries rate for the 6-, 7-, and 8-year-old children after 12 to 22 months of fluoridated water showed an increase in the rate of the deciduous teeth for the 6- and 8-year-old children while the rate for the 7-year-olds was decreased. A reduction was shown in the decayed, missing and filled rate of the permanent teeth of the 6-, 7-, and 8-year-olds.

The current report is based on the caries rates of the 12-, 13-, and 14-year-old children in Evanston after 23 to 34 months' exposure to fluoridated drinking water.

FINDINGS

The 1949 survey indicated a reduction of approximately 12 per cent in the caries experience of the permanent teeth of 12-, 13-, and 14-year-old school children when compared to the base examinations of students of the same age groups. The children demonstrating immune permanent teeth increased about 73.21 per cent for the three age groups.

The precarious lesions were markedly reduced. Precarious lesions being those deep pits, fissures, and grooves where a questionable etching along the peripheral border was present, or questionable soft material was demonstrable at the base of the pit, fissure, or groove, and yet where there was no frank caries present. (Tables I, II, and III.)

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TABLE I

CARIES RATES OF 12-YEAR-OLD EVANSTON CHILDREN IN 1946 AND 1949

	1946 NaF 0.0	1949 NaF 1.0	PERCENTAGE
Examinations	418	522	
D.M.F. permanent teeth	763.16	681.99	R 11.16
Permanent molars occlusal surface caries experience	351.67	313.41	R 10.88
Free from occlusal surface caries experience	194.02	221.46	I 14.14
D.F. anterior teeth (cuspid to cuspid)			
Maxillary	127.27	110.34	R 13.30
Mandibular	31.82	28.16	R 11.50
Tooth surfaces with precarious lesions (deep pits and fissures)	130.62	57.85	R 55.71
Immunity to dental caries	1.44	1.53	I 6.25

Fluoridation period is 23 to 34 months.
Rates are per 100 children.
R = Reduction.
I = Increase.

Comparison of the caries rates of all children in the study area (Evanston, Ill.) and the control area (Oak Park, Ill.) prior to the addition of sodium fluoride to the communal water supply of the study area indicated a lower caries rate for school children of the control area.

In an effort to find the source of these differences in caries prevalence, it was found to be due largely to differences in the make-up of the student groups examined in the two areas. While in the study area 22.2 per cent of the children examined were attending parochial schools, no such children were included in the control area: and while 5.6 per cent of the children in the study area were Negro children, only 0.1 per cent of the children in the control area were Negro. Statistically significant differences were found to exist between the caries rates of Negro and parochial school children on one hand, and public white school children on the other hand.

Generally, the caries rates of parochial school children were found to be higher and those of Negro children lower than those of white children in public schools. Therefore, comparisons of caries rates for the study group and

TABLE II

CARIES RATES OF 13-YEAR-OLD EVANSTON CHILDREN IN 1946 AND 1949

	1946 NaF 0.0	1949 NaF 1.0	PERCENTAGE
Examinations	688	677	
D.M.F. permanent teeth	1009.59	857.02	R 15.11
Permanent molars occlusal surface caries experience	438.08	364.99	R 16.6
Free from occlusal surface caries experience	190.84	253.91	I 33.04
D.F. anterior teeth (cuspid to cuspid)			
Maxillary	157.26	135.60	R 13.77
Mandibular	45.20	35.75	R 20.91
Tooth surfaces with precarious lesions (deep pits and fissures)	139.10	85.52	R 38.80
Immunity to dental caries	1.02	2.51	I 146.08

Fluoridation period is 23 to 34 months.
Rates are per 100 children.
R = Reduction.
I = Increase.

TABLE III

CARIES RATES OF 14-YEAR-OLD EVANSTON CHILDREN IN 1946 AND 1949

	1946 NaF 0.0	1949 NaF 1.0	PERCENTAGE
Examinations	595	570	
D.M.F. permanent teeth	1165.88	1086.84	R 6.78
Permanent molars occlusal surface caries experience	471.26	406.49	R 13.74
Free from occlusal surface caries experience	183.02	238.25	I 30.18
D.F. anterior teeth (cuspid to cuspid)			
Maxillary	172.60	153.56	R 11.03
Mandibular	45.71	38.25	R 16.71
Tooth surfaces with precarious lesions (deep pits and fissures)	139.33	92.81	R 33.38
Immunity to dental caries	1.01	1.75	I 42.20

Fluoridation period is 23 to 34 months.
Rates are per 100 children.
R = Reduction.
I = Increase.

the control group are based on the caries experience of public white school children only, while such comparisons involving children in only the study area are based on the caries experience of all children in total. The caries rates for the Evanston white school children in the 1946 survey and the Oak Park white school children in the 1947 survey were very similar.

It is interesting to note that the 1949 survey, when compared with the base line of 1946, indicated a lowering of the rates for the public and parochial 12-, 13-, and 14-year-old white children and a slight increase of the rate for the Negro children in Evanston when examined after 23 to 34 months of fluoridated water (Table IV).

In further comparing the rates for Oak Park (control) and Evanston (study area) it is apparent that the base line figures are very similar. However, in 1949 the Evanston group demonstrated reduced rates for the occlusal surface caries experience, decayed and filled anterior teeth and tooth surfaces with precarious lesions, after 23 to 34 months of fluoridated water. The 14-year-old children in the Evanston 1949 examinations were the only children to carry permanent tooth immunity rates greater than the 1946 base line group and the 1947 control group. The 12- and 13-year-old groups of 1949 were below one or the other or both of the same groups in the base line examinations (Tables V, VI and VII).

TABLE IV

D.M.F. PERMANENT TEETH RATES OF 12- 13- AND 14-YEAR-OLD EVANSTON AND OAK PARK CHILDREN

	EVANSTON 1946			OAK PARK 1947
AGE	PUBLIC SCHOOL (WHITE CHILDREN)	FOSTER SCHOOL (NEGRO ONLY)	PAROCHIAL SCHOOL CHILDREN	PUBLIC SCHOOL (WHITE ONLY)
12	707.51	658.82	981.32	774.29
13	946.17	861.76	1218.13	970.00
14	1133.33	1035.71	1322.22	1194.64
	EVANSTON 1949 (23 TO 34 MONTHS OF FLUORIDATED WATER)			
12	625.49	697.14	826.86	
13	785.89	1127.50	1008.38	
14	1073.31	1080.00	1129.85	

Rates are per 100 children.

TABLE V

CARIES RATES OF 12-YEAR-OLD EVANSTON AND OAK PARK CHILDREN

	OAK PARK 1947 NaF 0.0	EVANSTON 1946 NaF 0.0	EVANSTON 1949 NaF 1.0
Examinations	280	293	353
D.M.F. permanent teeth	774.29	707.51	625.49
Permanent molars occlusal surface caries experience	362.86	337.20	308.21
Free from occlusal surface caries experience	223.93	204.10	238.52
D.F. anterior teeth (cuspid to cuspid)			
Maxillary	122.14	112.29	97.73
Mandibular	26.07	31.74	21.53
Tooth surfaces with precarious lesions (deep pits and fissures)	107.14	139.59	60.34
Immunity to dental caries	1.79	1.71	1.70

Fluoridation period in Evanston 23 to 34 months.
Public white school children only.
Rates are per 100 children.

In comparing the findings of Dean, Arnold, Jay and Knutson for Aurora (1945 and 1946), where fluorine is a natural portion of the communal water and where only life time resident children were examined, to Evanston after 23 to 34 months of artificially fluoridated water, we find a considerably lower caries rate for the Aurora children (Table VIII).

As radiographs were employed in the current Evanston study it is apparent that early proximal caries would be disclosed to a greater extent than in the previous surveys. We find our base line figures for caries experience in Evanston and Oak Park approximately 32 per cent higher than those of Dean and his co-workers for Evanston and Oak Park in 1941. We assume this may be explained partially by differences in the techniques of examination, particularly in the use of x-ray in the current investigation.

DISCUSSION

The reduction in dental caries experience rates in Evanston is quite similar for all age groups. The percentage reduction in D.M.F. permanent teeth is highest among the 13-year-old children. Since the second permanent

TABLE VI

CARIES RATES OF 13-YEAR-OLD EVANSTON AND OAK PARK CHILDREN

	OAK PARK 1947 NaF 0.0	EVANSTON 1946 NaF 0.0	EVANSTON 1949 NaF 1.0
Examinations	404	483	482
D.M.F. permanent teeth	970.00	946.17	785.89
Permanent molars occlusal surface caries experience	403.22	426.50	356.84
Free from occlusal surface caries experience	234.16	206.41	208.09
D.F. anterior teeth (cuspid to cuspid)			
Maxillary	132.18	139.54	123.03
Mandibular	37.38	37.68	31.12
Tooth surfaces with precarious lesions (deep pits and fissures)	101.24	152.80	84.85
Immunity to dental caries	2.48	1.24	2.28

Fluoridation period in Evanston 23 to 34 months.
Public white school children only.
Rates are per 100 children.

TABLE VII

CARIES RATES OF 14-YEAR-OLD EVANSTON AND OAK PARK CHILDREN

	OAK PARK 1947 NaF 0.0	EVANSTON 1946 NaF 0.0	EVANSTON 1949 NaF 1.0
Examinations	336	450	416
D.M.F. Permanent Teeth	1194.64	1133.33	1073.31
Permanent molars occlusal surface caries experience	445.83	467.11	411.53
Free from occlusal surface caries experience	210.12	196.44	244.71
D.F. anterior teeth (cuspid to cuspid)			
Maxillary	187.51	162.67	153.85
Mandibular	39.88	41.56	32.69
Tooth surfaces with precarious lesions (deep pits and fissures)	94.05	153.33	92.55
Immunity to dental caries	1.19	0.89	1.92

Fluoridation period in Evanston 23 to 34 months.
Public white school children only.
Rates are per 100 children.

molar erupts at 12 years we may assume that at 13 years of age the length of exposure to caries attack is approximately 1 year, which probably is not sufficient time to find caries established in the second molars, particularly in the presence of fluoride-containing water.

The occlusal surface caries experience of the permanent molars of the 12-, 13-, and 14-year-old children indicated a reduced caries rate accompanied by a slight increase in the occlusal surfaces free from caries experience. Again the greatest change was associated with the 13-year-old group.

The reduction of precarious lesions, quite apparent in the 12-, 13-, and 14-year-olds, may be attributed partially to the time of the eruption period of some of the permanent teeth. At the time fluorine was placed in the water, 2 years previous to this examination the 12-, 13-, and 14-year-olds were then 10, 11, and 12 years of age respectively. As the cuspids, the first and second bicuspids, and the second molars, usually erupt during the tenth, eleventh, and twelfth year, the enamel of some of these teeth has been exposed to fluorine in the tissue fluids postformative but pre-eruptive and erupted into the mouth fol-

TABLE VIII

COMPARISON OF D.M.F. CARIES RATES OF THE PERMANENT TEETH OF LIFE TIME RESIDENT WHITE SCHOOL-CHILDREN OF AURORA

				EXAMS.	YEARS OF AGE 12	13	14
	Aurora (1945-46) entire life	F	1.2 ppm	1235	295	309	364
Dean*	Evanston (1941)	F	0.0 ppm	673	563	676	793
	Oak Park (1941)	F	0.0 ppm	329	583	696	907
	Evanston (1946)	F	0.0 ppm	1226	707.51	946.17	1133.33
EDCS‡	Oak Park (1947)	F	0.0 ppm	1020	747.51	970.00	1194.64
	Evanston (1949)	F	1.0 ppm†	1251	625.49	785.89	1073.31

Fluorine 1.2 ppm naturally.
Oak Park, 1941, Survey and Evanston, 1941, Survey compared to Evanston, 1946, Oak Park, 1947, and Evanston, 1949.
*From Dean's Published Reports.
†Fluorine 23 to 34 months.
‡Evanston Dental Caries Study.

lowing the introduction of sodium fluoride to the communal water supply. We are permitted to assume that the reduced number of precarious lesions may be accounted for by the action of the fluoridated water on the enamel surfaces of these teeth, or that the exposure time to destructive mouth acids, if present and uninhibited, is too short to produce caries at this time.

SUMMARY

The exposure time of the 12-, 13-, and 14-year-old children to sodium fluoride has been for 23 to 34 months. The rates show a reduction of 12.19 per cent for these children in the decayed, missing and filled permanent teeth.

The precarious lesions of the permanent teeth were reduced by 55.7 per cent for the 12-year-olds, 38.80 per cent for the 13-year-olds and 33.3 per cent for the 14-year-olds. The 13-year-old children demonstrated the greatest increase in immune cases, that of 146.0 per cent. An increase of that amount appears rather staggering but when considering the actual number of immune cases per 100 children, 1.02 in 1946, the increase to 2.51 in 1949 is only 1.49 cases per 100 children.

The lowered caries rate for Evanston in 1949 is apparently attributed to the fluoridated water. There is a reduction in the dental caries experience of the permanent teeth of all three age groups. This reduction may be due partially to the very widespread use of the urea dentifrices. Topical application of sodium fluoride is now given to many patients. We have made a determined effort to have accurate records of its use in Evanston. There may be a slight inclination toward better oral hygiene by the students as they become acquainted with the study by our frequent visits in the schools. However, until a definite percentage reduction of the caries rate of the study area is established that is materially less than the prevailing caries rate of the control area, where we know that topical application of sodium fluoride is given and urea dentifrices probably are used to about the same extent as in Evanston, we feel that this reduction shown here may not be assumed to be due entirely to the action of sodium fluoride.

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THE EVANSTON DENTAL CARIES STUDY

VIII. Fluorine Content of Vegetables Cooked in Fluorine Containing Waters

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AS A part of the Evanston Dental Caries Study, an investigation has been made for the purpose of providing information regarding the probable amount of fluorine ingested in the complete diet. The first phase of this project has been primarily concerned with the vegetables included in the daily diet. The results presented here are considered worthy of publication because they represent recent analyses by an accepted method.

A search of the literature has shown that the published results for foods cooked in water containing fluorides are incomplete. Smith, Smith, and Vavich[1] have indicated that the fluorine content of foods may be increased when cooked in fluoride waters.

METHODS

General: In August, 1947, a questionnaire was sent to 15 grocery stores (independent and chain) representing all sections of the City of Evanston to determine which type of vegetable (fresh, canned, or frozen) sells in greatest quantity, and which varieties of vegetables in each category are in greatest demand. The answers to these questions were given on a yearly basis, and from this survey the vegetables were selected for analysis in the laboratory. The vegetables used were either purchased on the open market or grown in gardens in Evanston. To simulate methods of cooking in the home, duplicate batches of the vegetables were cooked in a saucepan and pressure cooker. The amount of water used in the cooking process was kept constant for all of the vegetables. When using the saucepan method, 200 ml. of water were used; for the pressure cooker method, 100 ml. of water were used. A cook book was referred to for the cooking time of each vegetable.

To determine the extent of absorption of fluorine by foods, each vegetable was divided into three lots. The first lot was analyzed for its fluorine content in the fresh raw state; the second lot was divided into four equal portions which were then boiled in distilled water containing 0.0, 1.0, 2.0, and 5.0 parts per million of fluorine; the third lot, also divided into four equal portions, was pressure cooked using distilled water containing 0.0, 1.0, 2.0, and 5.0 parts per million of fluorine. After cooking, the liquid was drained off and the vegetables were washed with distilled water. The cooked foods were than analyzed for their fluorine content, and in many cases the cooking liquid was also analyzed for its fluorine content.

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The method of analysis used was that of Willard and Winter[2] using the modifications of the Association of Official Agricultural Chemists,[3] with magnesium acetate being employed as the fluorine fixative. The distillation apparatus is of Pyrex glass.* All precautions were taken to maintain a minimum distillation blank, including the use of platinum dishes when ashing the samples.

RESULTS AND OBSERVATIONS

A summary of the questionnaire sent to the 15 grocery stores in Evanston is shown in Table I. These data have been prearranged to show the types of foods sold in greatest quantity and the kinds of vegetables in greatest demand. It was from this table that the vegetables were selected for use in this Study.

TABLE I

EVANSTON FOOD SURVEY

1. Types of food selling in greatest quantity
   a. Fresh
   b. Canned
   c. Frozen
2. Kinds of vegetables in greatest demand
   a. Fresh
      1. Carrots
      2. Beans
      3. Cauliflower
      4. Peas
      5. Tomatoes
      6. Spinach
      7. Cabbage
      8. Corn
   b. Canned
      1. Peas
      2. Corn
      3. Tomatoes
      4. Beans
      5. Spinach
   c. Frozen
      1. Peas
      2. Beans
      3. Spinach
      4. Corn
      5. Asparagus
      6. Broccoli

Fresh Vegetables.—Table II is a composite of all the results obtained by cooking fresh vegetables in a saucepan or pressure cooker. This table shows that the foods cooked in a pressure cooker absorb a smaller amount of fluorine than those cooked in a saucepan. The increase in the fluorine content of the leafy vegetables after cooking in a saucepan is somewhat greater than the increase found for the less penetrable beets and carrots, but when the pressure cooker was used, there was not an appreciable difference. An important observation is that the amount of fluorine absorbed by the vegetables is proportionately less in water containing higher concentrations of fluorine, as compared to water containing lower concentrations of the chemical.

*All glass connections as recommended by the Associate Referee of the Food and Drug Administration, Washington, D. C.

TABLE II

THE COMPARATIVE FLUORINE CONTENT OF FRESH VEGETABLES COOKED IN WATER WITH AND WITHOUT FLUORINE

	FLUORINE CONTENT (PARTS PER MILLION)								
		BOILED IN SAUCEPAN P.P.M. F IN WATER				PRESSURE COOKED P.P.M. F IN WATER			
VEGETABLE	RAW	0.0	1.0	2.0	5.0	0.0	1.0	2.0	5.0
Carrots	0.14	0.17	0.81	1.75	3.61	0.18	0.51	0.69	1.02
Beans	0.20	0.21	0.96	1.72	4.32	0.19	0.52	0.67	1.23
Cauliflower	0.27	0.27	1.24	2.10	5.03	0.27	0.69	1.09	2.24
Peas	0.22	0.28	1.22	2.02	3.88	0.25	0.84	1.08	1.52
Spinach	0.84	1.00	2.02	2.85	4.99	0.76	1.13	1.63	2.81
Cabbage	0.23	0.29	1.13	1.88	4.92	0.23	0.55	0.79	1.03
Beets	0.21	0.26	0.60	1.16	1.88	0.28	0.44	0.57	0.78
Tomatoes	0.17	0.23	0.61	–	–	0.13	0.26	–	–
Corn (cob)	0.24	0.29	0.55	–	–	0.17	0.42	–	–

All results are averages based upon the fresh weight.

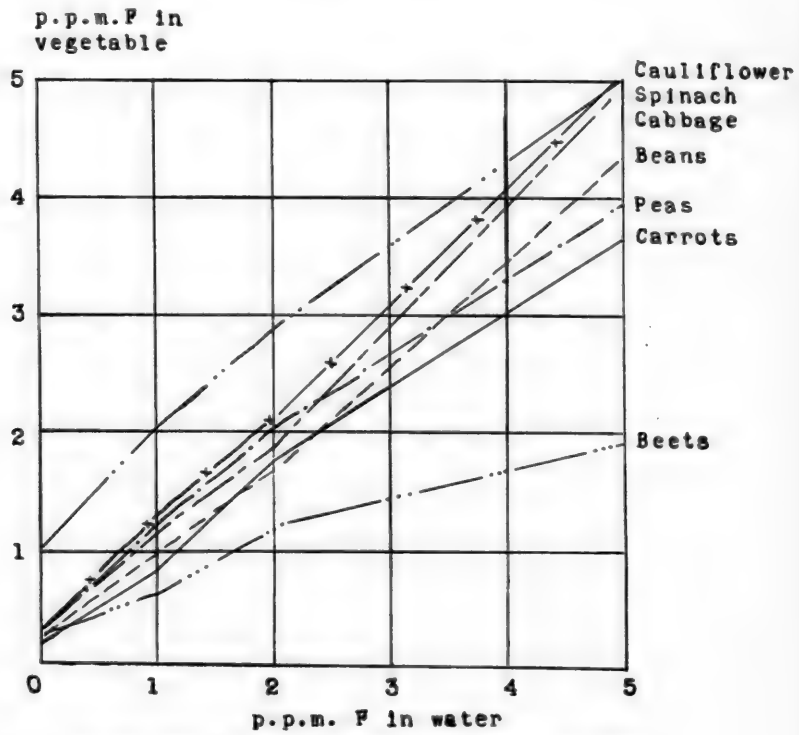

Fig. 1.—Absorption of fluorine by vegetables cooked in a saucepan.

Key: Carrots ————————
Beans — — — — —
Peas —— . —— . ——
Spinach —— .. —— .. ——
Beets —— ... —— ...
Cabage —— - —— - ——
Cauliflower —— x —— x

Fig. 1, which is for the vegetables cooked in a saucepan, and Fig. 2, which is for the pressure cooked vegetables, substantiate the previous statements. Figs. 1 and 2 are constructed from the results shown in Table II, to graphically illustrate the absorption of fluoride by vegetables cooked in fluoride containing waters.

Frozen Vegetables.—Due to the increasing popularity of frozen vegetables in the daily diet, several were included in this study. The frozen vegetables were cooked and analyzed in the same manner as the fresh foods. In this class the vegetables selected were limited to the three top sellers—beans, peas, and spinach. The results of the analyses of the frozen foods as well as a comparison of them with the fresh vegetables are shown in Table III. A study of this

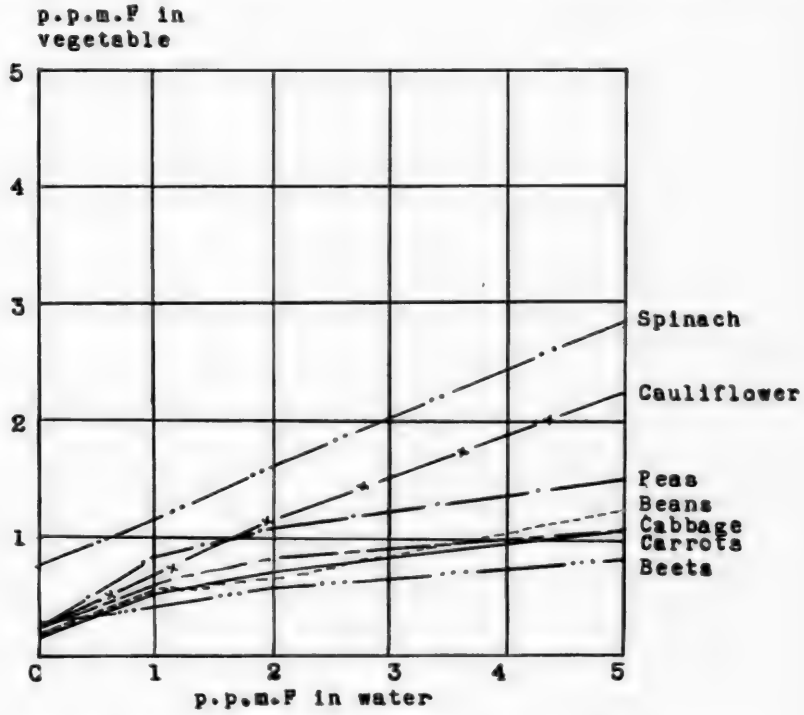

Fig. 2.—Absorption of fluorine by vegetables cooked in a pressure cooker .

table reveals the fact that the frozen vegetables appear to absorb more fluorine than fresh vegetables from water containing 5.0 parts per million of fluorine. This applies to both methods of cooking. In concluding these statements pertaining to the fresh and frozen vegetables, it may be said that when the cooking liquids were analyzed, the amount of fluorine remaining in the liquid plus the fluorine absorbed by the vegetables totaled 95 to 100 per cent of the amount of fluorine added at the start of the cooking process.

Canned Vegetables.—Canned vegetables were not included in this report because of the uncertainty of the water supplies at the canneries. At the pres-

TABLE III

COMPARATIVE FLUORINE CONTENT OF FRESH AND FROZEN VEGETABLES—RAW AND COOKED

VEGETABLE		FLUORINE CONTENT IN P.P.M.								
		RAW	BOILED IN SAUCEPAN				PRESSURE COOKED			
			0.0	1.0	2.0	5.0	0.0	1.0	2.0	5.0
Beans	Fresh	0.20	0.21	0.96	1.72	4.32	0.19	0.52	0.67	1.23
	Frozen	0.20	0.22	1.21	2.16	4.98	0.21	0.77	1.49	2.63
Peas	Fresh	0.22	0.28	1.22	2.02	3.88	0.25	0.84	1.08	1.52
	Frozen	0.30	0.28	1.15	2.04	4.56	0.23	0.58	1.10	1.88
Spinach	Fresh	0.84	1.00	2.02	2.85	4.99	0 76	1.13	1.63	2.81
	Frozen	0.28	0.25	1.01	1.74	4.52	0.23	0.69	1.19	1.90

All results are averages based upon the fresh weight.

ent time we are primarily concerned with the absorption of fluorine by vegetables in the cooking process where the fluorine content of the water is definitely known.

TABLE IV

COMPARISON OF RAW FRESH VEGETABLES
EVANSTON AND LITERATURE

VEGETABLE	FLUORINE CONTENT IN P.P.M.	
	EVANSTON	LITERATURE
Carrots	0.14	0.20
Beans	0.20	0.13
Cauliflower	0.27	0.12
Peas	0.22	0.60
Tomatoes	0.17	0.24
Spinach	0.84	1.00
Cabbage	0.23	0.30
Corn	0.24	0.20
Beets	0.21	0.20

All results are based upon the fresh weight.

COMPARISONS WITH LITERATURE

In reviewing the literature, a number of publications were found showing the fluorine content of fresh raw vegetables.[4-9] A comparison of these data with the results of this study is shown in Table IV. It will be noted that for most of the vegetables there is a similarity of results.

Table V is a comparison of results found in this study with those previously reported in literature for vegetables boiled in fluoride containing

TABLE V

COMPARISON OF BOILED FRESH VEGETABLES
EVANSTON AND LITERATURE

VEGETABLE	P.P.M. FLUORINE IN BOILED VEGETABLES			
	AS FOUND IN EVANSTON		AS FOUND IN LITERATURE	
	BOILED IN F FREE H_2O	BOILED WITH 5.0 P.P.M. F IN H_2O	BOILED IN F FREE H_2O	BOILED WITH 5.0 P.P.M. F IN H_2O
Carrots	0.17	3.61	0.0	3.5
Beans	0.21	4.32	–	–
Peas	0.28	3.88	–	–
Spinach	1.00	4.99	2.0	4.0
Cabbage	0.29	4.92	0.0	3.6
Beets	0.26	1.88	0.0	1.0

water. In this case, however, there was only one publication[1] available for reference, which shows the need for more work in this field. The figures shown in Table V do not have the same analogy as those in Table IV.

SUMMARY AND CONCLUSIONS

1. Vegetables cooked in fluoride-bearing waters do absorb fluorine in proportion to the fluorine content of the water.

2. Vegetables cooked in a saucepan absorb more fluorine than vegetables cooked in a pressure cooker.

3. Frozen vegetables absorb more fluorine than fresh vegetables when cooked in water containing 5.0 parts per million of fluorine, but little difference is noticed when the fluoride concentration of the water is 1.0 and 2.0 parts per million.

4. Vegetables cooked in fluoride-bearing waters have a direct effect upon the daily dietary fluorine supplement.

The author wishes to express his appreciation to Dr. J. R. Blayney and Dr. W. H. Tucker for their helpful suggestions and criticisms.

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A SELECTIVE MEDIUM FOR THE ISOLATION AND ENUMERATION OF ORAL LACTOBACILLI

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IT WOULD be highly desirable for those studying the role of lactobacilli in dental caries to have a medium that is selective for oral lactobacilli and which favors their good growth. The Hadley[3] tomato juice agar modified by Jay and Arnold,[4] henceforth mentioned as TJ agar, has been employed widely and is established as a valuable tool in the hands of experienced workers. However, it has been generally recognized that this medium has certain limitations. While lactobacilli may be differentiated from other microorganisms on this medium, it is not entirely selective for lactobacilli since yeasts, micrococci, streptococci, molds, gram-negative rods, sporeforming bacilli, etc., may occur with varying frequency. Thus, it may be difficult and time consuming to differentiate the lactobacilli from some of the other organisms. In the case of animal samples, plates obscured by spreading growth sometimes occur with sufficient frequency to interfere seriously with an experimental program.

This paper will describe a selective medium for the isolation and enumeration of oral lactobacilli from man and experimental animals.

PLAN OF EXPERIMENTS

Tomato juice agars have been employed extensively as highly favorable media for lactobacilli. Therefore, many preliminary experiments, including a number with sodium azide, were conducted to modify TJ agar. Diamond[2] had used sodium azide in Hadley[3] TJ agar to suppress much of the extraneous flora. It was found in the present authors' experiments that this procedure was unsatisfactory since many lactobacilli were inhibited (Lichstein and Soule[5] and Rogosa and Mitchell[6]).

The authors were unsuccessful in developing an adequate synthetic medium since some lactobacilli could not be grown satisfactorily on such substrates. However, it soon became evident, in the analysis of approximately 2,000 human and animal oral samples, that the control of the concentrations of phosphate, citrate, acetate, salts, sorbitan monooleate, and hydrogen ions in an otherwise nutritionally adequate environment offered a promising approach to the development of a satisfactory selective medium for oral lactobacilli. The composition of the resulting medium, henceforth noted as SL agar, was as follows:

Trypticase (BBL)	10 Gm.
Yeast Extract (Difco)	5 Gm.
KH_2PO_4	6 Gm.
Ammonium Citrate [$(NH_4)_2HC_6H_5O_7$]	2 Gm.
Salt Solution†	5 ml.

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†Salt Solution: $MgSO_4.7H_2O$, 11.5 g; $MnSO_4.2H_2O$, 2.4 g *or* $MnSO_4.4H_2O$, 2.86 g; $FeSO_4.7H_2O$, 0.68 g; distilled H_2O to 100 ml.

Glucose	20 Gm.
Sorbitan Monooleate*	1 Gm.
Sodium Acetate ($NaC_2H_3O_2 \cdot 3H_2O$)	25 Gm.
Glacial Acetic Acid (99.5%)	1.32 ml.
Agar	15 Gm.
Distilled H_2O to 1 liter; pH 5.40	

Preparation of SL Agar.—The sorbitan monooleate was dissolved in distilled water to make a stock solution containing 100 mg. per ml.; 10 ml. was then used per liter of medium. The salt solution may settle out on standing and may be resuspended by shaking. If the phosphate, citrate, acetate, salt solution, sorbitan monooleate, and acetic acid are dispensed accurately the pH will be 5.40 without further adjustment.

In the preparation of 1 liter of medium, the agar was dissolved separately from the other ingredients in 400 ml. of distilled water. The remaining ingredients were also dissolved separately in 400 ml. of distilled water. This latter solution was brought to a boil and added to the hot solution of agar. The volume was adjusted to 1 liter with distilled water, the medium was dispensed, and immediately sterilized no longer than 10 minutes at 15 pounds in the autoclave.

All the human salivary specimens were plated on the medium prepared and sterilized as described above.

Prolonged or repeated heating of the medium tends to darken it and if this has occurred some types of lactobacilli, present in a small percentage of samples, may not develop to full size but rather present the appearance of pin-point colonies. Since sporeforming or other heat-resistant organisms had not grown in SL agar in repeated tests, it seemed as if autoclave sterilization was unnecessary. Therefore, at the time of preparation, the hot (at least 80° C.) medium was dispensed repeatedly into clean, nonsterile flasks which were then plugged with cotton. The SL agar did not become contaminated during the experimental holding period of 2 weeks at room temperature or for at least 6 months in the refrigerator.

All the animal samples were plated on the SL agar which had not been autoclaved. The medium thus prepared appeared clear and practically colorless in the plates. The authors are now routinely following this procedure.

EXPERIMENTS WITH PURE CULTURES OF ORAL LACTOBACILLI

Unless it could be determined that a comprehensive spectrum of representative pure cultures of oral lactobacilli could be grown favorably on SL agar, the usefulness of the medium could be questioned. Therefore, the strains noted in Table I, which were originally isolated from TJ agar, were grown in a broth of the same composition as the TJ agar but with the agar omitted. These strains were pour plated in duplicate in TJ and SL agars both of which were at pH 5.4. In some cases TJ agar at pH 5.0 was also used since the oral samples discussed later were all plated on the latter medium. It is evident from the representative data in Table I that the numbers of lactobacilli were generally comparable on the two media. However, the size and rate of development of the colonies were often greater in SL agar.

*Sorbitan monooleate "Tween 80," obtained from the Atlas Powder Company, Wilmington, Delaware.

Sampling from Human Saliva.—Approximately 5 ml. of paraffin-stimulated saliva were collected in a screw-cap sterile bottle and shaken by machine for two minutes. The saliva was diluted 1:100 by transferring 1 ml. to 99 ml. distilled water in a 6 ounce screw-cap prescription bottle, and shaken. One plate was inoculated with 1 ml. and another plate with 0.1 ml. from the 1:100 dilution. The use of milk dilution pipettes graduated at 1.0 and 1.1 ml. facilitated this operation. About 20 ml. of medium were poured into the plates and mixed with the inoculum. After the medium had solidified, the inverted plates were incubated for 3 days at 37° C.

TABLE I. COMPARATIVE GROWTH OF PURE CULTURES OF LACTOBACILLI IN TJ AND SL AGARS

ORGANISM	TJ AGAR (pH 5.0) NO. × 10^7/ML.	TJ AGAR (pH 5.4) NO. × 10^7/ML.	SL AGAR NO. × 10^7/ML.
L. casei No. 541	95	92	93
L. casei No. 167	148	176	158
L. casei No. 19	236	286	282
L. casei No. 303	–	109	132
L. casei No. 337	–	169	176
L. casei No. 879A	94	87	89
L. casei No. H110	–	106	94
L. casei No. H111	–	86	104
L. casei No. H221	–	25	18
L. casei No. Ld 1	–	111	117
L. fermenti No. 11	54	48	49
L. fermenti No. H219	–	113	136
L. fermenti No. H505	–	33	48
L. fermenti No. 69	–	192	119
L. fermenti No. 70	–	275	245
L. fermenti No. 20	–	49	142
L. acidophilus No. Ld18	–	<1	16
L. acidophilus No. Ld23	–	18	20
L. brevis (II) No. 619	177	264	183
L. brevis (II) No. 737	379	328	358
L. plantarum No. 858	82	101	69
L. heterofermentative sp. No. 4B	132	153	145
L. heterofermentative sp. No. 223	–	440	480
L. heterofermentative sp. No. H 505	–	33	48
L. heterofermentative sp. No. Ld 13	–	423	442

In the case of most human salivary samples, all the colonies on at least one of the plates could be counted with the aid of a colony counter. Exceptions to this were those few instances in which the plates of the 1:100 dilution were crowded. Since the distribution of the colonies in the poured plates of SL agar was usually uniform, the numbers of lactobacilli in these crowded plates were estimated rather well by counting the colonies present in representative areas either with the aid of a colony counter or a wide field microscope at a magnification of 19.5. The final count was expressed as numbers of lactobacilli per ml. of saliva.

The corresponding samples, which were plated on SL agar as described above, were also plated on one lot of Hadley (1933) TJ agar as prepared by the National Institutes of Health modification of Jay and Arnold (1946). The plates were inoculated by the Hadley[3] streak technique and incubated 4 days at 37° C. The counts were made by estimating the numbers of organisms present in at least six selected areas which, in the judgment of the operator, were representative. A wide field microscope was employed for this purpose.

Many of the TJ agar plates were counted independently by two of the authors and in addition were sometimes recounted by the same operator without knowledge of the count previously obtained. The deviations of the counts obtained in these tests were such that it did not seem sound to compare SL agar quantitatively with TJ agar within ± 50 per cent, "especially in view of the statistical significance of errors in counts where 100,000 lactobacilli per c.c. of saliva or more are recorded" (Snyder[7]). Furthermore, different dilutions were employed with the corresponding human samples on the two media. For these reasons, the counts on the two media are compared later within the range of 100 per cent differences.

Sampling from Hamsters.—Two different procedures were used for sampling hamsters. (1) In order to obtain a sample from the buccal pouch, a sterile swab was inserted to the depth of the pouch and rotated 5 times. The stick of the swab was cut above the cotton and dropped into a tube containing 5 ml. of a 0.03 per cent yeast extract broth. The tubes were permitted to remain at room temperature for about an hour and then shaken before samples were withdrawn for analysis. (2) The mouth samples were obtained by rotating a swab over the accessible surfaces of the teeth and soft tissues. It was necessary to prepare the plates at dilutions of 1:1,000 and 1:10,000 to obtain satisfactory results from many of the hamster samples. The corresponding samples were all plated in the same dilution on both SL and TJ agars, using the techniques as described under human sampling. The counts were expressed as numbers of lactobacilli per swab.

Examination of the Plates.—In order to determine whether only lactobacilli were growing in SL agar, five or more representative colonies were smeared from each SL agar plate and examined microscopically. Ten or more smears were made from at least half of the plates and in some cases every colony on the SL agar plates was smeared. Frequent isolations were made from the SL agar plates and the isolates were studied to determine if they were lactobacilli as described in Bergey's Manual.[1] In addition, smears of representative colonies were made from the TJ agar plates and all catalase-negative, gram-positive, nonsporulating, rods were counted as lactobacilli.

RESULTS FROM HUMAN SAMPLES

The comparative results from 122 human salivary samples, in which each sample was plated in SL agar and on TJ agar, are noted in Table II. These samples comprised 23 children's and 99 adult salivary specimens. It is evident that the numbers of lactobacilli in SL agar compared favorably, within the limits of experimental variation, with the numbers of lactobacilli on corresponding plates of TJ agar.

Forty-one (33.6 per cent) of the 122 samples yielded counts on SL agar greater by 100 per cent than those on TJ agar; sixty-seven (54.9 per cent) were comparable on the two media; twelve (9.8 per cent) of the cases could not be compared because of the unsatisfactory character of the TJ agar plates. These 12 plates were covered with molds or other confluent, extraneous growth, or else the distribution of the surface inoculum was so inhomogeneous that a count

TABLE II. COMPARATIVE DATA FROM HUMAN SALIVARY SAMPLES IN SL AGAR AND ON TJ AGAR*

	SL AGAR		TJ AGAR			SL AGAR		TJ AGAR	
	LACTOBACILLI	OTHER ORGANISMS	LACTOBACILLI	OTHER ORGANISMS		LACTOBACILLI	OTHER ORGANISMS	LACTOBACILLI	OTHER ORGANISMS
SAMPLE NO.	NUMBER/ML. × 1000		NUMBER/ML. × 1000		SAMPLE NO.	NUMBER/ML. × 1000		NUMBER/ML. × 1000	
1	0	2.4	0	2.4	51	43	0	35	strep.
2	46	0	21	0	52	2.6	0	1.8	0.25
3	2.2	0	1.5	0	53	37	0	15	2
4	281	0	60	mold	54	370	0	270	0.15
5	8.2	0	6	0	55	1560	0	750	5
6	4.7	0	1.75	0.5	56	932	0	500	10
7	105	0	42	mold	57	770	0	675	3
8	2.8	0	3	0	58	0.9	0.6	2	3.55
9	1350	0.2	270	2.8	59	0.1	0	0	8.8
10	780	0	6	0	60	1.3	0	1	0.55
11	19	0	6	0	61	51	0	30	2.5
12	3.7	0	0	0	62	361	0	324	mold
13	2	0	0	0	63	1800	0	860	0.05
14	229	0	12	0	64	12	0	15	2.5
15	490	0	378	2.45	65	277	0	54	0.05
16	3.45	0	0	0	66	1.2	0	0.5	0.5
17	198	0	108	0.05	67	2940	0	1350	0
18	215	0	45	0	68	1.2	0	0.5	3
19	5.7	0	0.5	0	69	110	0	18	0.15
20	3.2	0	1	0	70	281	0	81	8
21	30	0	16	0	71	0.2	0	0.05	0.1
22	12.5	0	7	1.2	72	1	0	0.3	1
23	312	0	108	0.2	73	1.3	0	4	0.25
24	32	0	35	0.3	74	2.7	0	1.3	0.6
25	414	0	120	0	75	295	0	300	2.3
26	607	0	210	0	76	5.4	0	6	1.1
27	42	0	0	0	77	2.6	0	0	0.75
28	91	0	65	0	78	35	0	24	0.2
29	0	0	0.05	0	79	95	0	74	0.35
30	275	0	162	0.05	80	800	0	272	mold
31	41	0	14	0.5	81	0.1	0	0	1.5
32	7.1	0	1.5	0.25	82	71	0	19	0.2
33	81	0	73	0.45	83	289	0	155	0
34	11	0	9	0	84	125	0	50	0
35	31	0	22	0.05	85	63	0	25	2
36	0.1	0	0.1	0	86	68	0	27	0
37	269	0	200	mold	87	482	0	108	0.1
38	57	0	45	0.9	88	760	0	459	0
39	236	0	135	0	89	0.2	0	0.5	0.05
40	680	0	203	5	90	77	0	30	0.15
41	2.9	0	0.7	0.2	91	73	0	45	0.25
42	123	0	80	1	92	310	0	200	16
43	18	0	15	0	93	488	0	360	1.4
44	14.5	0	10	2.8	94	221	0	226	0
45	0.2	0	0.05	0.35					
46	0.1	0	0	0.15					
47	520	0	600	0.35					
48	0.1	0	0	0.95					
49	37	0	33	0.15					
50	541	0.2	500	1.5					

*Sixteen samples negative for lactobacilli on both media and 12 samples in which the TJ plates were unsatisfactory are not included.

could not be made with assurance. In two cases (1.6 per cent) the counts on SL agar were less than 100 per cent of those on TJ agar. However, the respective counts were of the same order of magnitude, namely, 1,300 and 4,000; and 900 and 2,000.

Twenty-six of the TJ agar plates were negative. Seventeen of the corresponding plates of SL agar were also negative; of the remaining corresponding SL agar plates four were counted as 100; four ranged from 2,000 to 4,000; and one plate yielded a count of 42,000. These latter organisms, which were determined to be lactobacilli, were found to be incapable of growth on TJ agar at pH 5.0.

A large number (eighty-eight or 72.1 per cent) of the TJ agar plates contained organisms other than lactobacilli. Micrococci appeared on forty; yeasts (generally Candida) on thirty-eight; streptococci on eleven; molds on nine; and gram-negative rods on six of the TJ agar plates.*

This compared with 4 (3.3 per cent) of the SL agar plates in which the only extraneous organisms were yeasts present in significantly reduced numbers. These data are summarized in Table IV.

RESULTS FROM HAMSTER SAMPLES

Since the hamster has been employed as a promising experimental animal in the study of dental caries, it was decided to investigate the comparative efficiency of SL and TJ agars in the analysis of 156 samples from hamsters. The results of this experiment are summarized in Table III.

TABLE III. COMPARATIVE DATA FROM HAMSTER ORAL SAMPLES IN SL AGAR AND ON TJ AGAR

	NO. OF PLATES	PER CENT
SL agar counts > TJ agar counts*	52	33.3
SL agar counts ≈ TJ agar counts	67	42.9
Counts not comparable**	33	21.2
TJ agar counts > SL agar counts	4	2.6
Negative plates	0	0

*As noted earlier the data are compared within the range of 100 per cent differences since the errors involved in the comparison of the two different media and different techniques do not warrant a closer comparison.

**In these 33 cases the TJ agar plates were useless chiefly because of extraneous spreading growth obscuring the entire plate.

Fifty-two (33.3 per cent) of the hamster samples yielded counts on SL agar greater by 100 per cent than those on TJ agar; sixty-seven (42.9 per cent) of the plates were comparable on the two media; and thirty-three (21.2 per cent) of the TJ agar plates were useless because of overgrowths of molds and other spreading organisms covering the entire plate, or because of extremely poor distribution of the surface inoculum. In four (2.6 per cent) of the samples the counts on TJ agar were greater by 100 per cent than those on SL agar. The respective counts were 135,000 and 60,000; 270,000 and 51,000; 150,000 and 50,000; and 2,500 and 500. There were no negative plates on either medium with these hamster samples.

Organisms other than lactobacilli grew on eighty-two (52.6 per cent) of the TJ agar plates in this animal series. Molds appeared on 49; micrococci and streptococci on 39; yeasts on 9; spore-forming bacilli on 8; and gram-negative rods on 2 of the TJ agar plates.†

*It may be noted that the sum of the above organisms was greater than the total number of plates containing microorganisms other than lactobacilli since fourteen of the plates contained more than one type of extraneous organism.

†It may be of incidental interest that SL agar has been successfully employed (fecal samples) for the specific growth of fecal lactobacilli from hamsters. None of the 90 corresponding TJ agar plates was usable because of the presence of spreading growth completely obscuring the plates.

Only three (1.9 per cent) of the SL agar plates permitted the growth of organisms other than lactobacilli. These easily recognizable extraneous organisms were yeasts present in very greatly reduced numbers. These data are noted in Table IV.

TABLE IV. ORGANISMS OTHER THAN LACTOBACILLI FROM HUMAN AND HAMSTER SAMPLES IN SL AND ON TJ AGARS

	SL AGAR		TJ AGAR	
	NO.	PER CENT	NO.	PER CENT
Human Samples				
Total plates containing organisms other than lactobacilli	4	3.3	88	72.1
Plates containing micrococci	0	0	40	32.8*
Plates containing yeasts	4	3.3	38	31.1*
Plates containing streptococci	0	0	11	9.0*
Plates containing molds	0	0	9	7.4*
Plates containing gram-negative rods	0	0	6	4.9*
Hamster Samples				
Total plates containing organisms other than lactobacilli	3	1.9	82	52.6
Plates containing molds	0	0	49	31.4*
Plates containing cocci	0	1.9	39	25.0*
Plates containing yeasts	3	0	9	5.8*
Plates containing spore-forming bacteria	0	0	8	5.1*
Plates containing gram-negative rods	0	0	2	1.3*

*It may be observed that the sum of the percentages of organisms other than lactobacilli exceed the percentages of plates in which these organisms occurred since some plates contained more than one type of extraneous organism.

RESISTANCE OF SL AGAR TO CONTAMINATION

It can be seen from the data already presented that SL agar is extremely resistant to contamination by organisms other than lactobacilli. In order to corroborate this conclusion from the experiments with oral samples, a group of 150 strains comprising oral micrococci, streptococci, spore-forming bacteria, gram-negative rods, and miscellaneous unidentified bacteria, originally isolated from TJ agar, were inoculated in SL agar and incubated for extended periods. There was no visible growth or colonial development of these strains when the plates were examined periodically at magnifications of 45×. SL agar plates were exposed to the air for 24 hours, then covered and incubated at room temperature and 37° C. with the same negative results as above. Also, dust and floor sweepings were inoculated on SL agar plates, and incubated at various temperatures until the agar had dried sufficiently to crack. There was no detectable growth on these plates in these experiments.

DISCUSSION

Although it has been shown that SL agar is a highly desirable medium for oral lactobacilli, it should not be assumed that SL agar is altogether favorable for the growth of dairy lactobacilli. The medium requires some modification, particularly in the salt solution, for the good growth of such dairy types as *Lactobacillus bulgaricus, L. helveticus,* and *L. lactis.* These organisms have not yet been recovered from favorable media in 2,000 oral samplings and apparently do not become implanted in the mouth.

Except for the infrequent presence of yeasts in greatly reduced numbers, SL agar completely inhibited the remaining extraneous oral flora and also supported the favorable growth of oral lactobacilli. The few, infrequent yeasts were easily recognizable by their relatively large, white, and aerobic colonies. Finally, in the experience of the authors, SL agar was simple to prepare, reproducible, inexpensive, and supported the good growth of oral lactobacilli within 72 hours of incubation.

SUMMARY

A new, easily prepared, inexpensive, and reproducible medium is presented for the selective isolation and enumeration of oral lactobacilli from human and animal samples. In the comparative study of 122 human salivary specimens and 156 oral hamster samples in the new SL agar and on the modified Hadley TJ agar, it was found that SL agar was almost completely specific for lactobacilli and produced counts which compared favorably witih those obtained from TJ agar. Except for the very infrequent presence of yeasts in greatly reduced numbers, all of the organisms other than lactobacilli found on TJ agar, such as micrococci or staphylococci, streptococci, molds, spore-forming bacteria, and gram-negative rods, are eliminated in SL agar.

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A HISTOLOGICAL STUDY OF THE EFFECT OF EARLY PHASES OF CARIES ON THE ORGANIC COMPONENT OF THE ENAMEL OF THE SYRIAN HAMSTER

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INTRODUCTION

ALTHOUGH dental caries is considered to occur in various laboratory animals, the histopathology of the early microscopic phases of the disease in the enamel has been given very little attention. Keyes[1] studied caries lesions in the Syrian hamster and showed, by histological sections of decalcified material, that the lesion in this animal appears quite similar to that described in humans. He observed a penetrating yellow-brown to brownish-gray discoloration of the enamel to be associated with early caries, but stated that these incipient changes were often difficult to identify positively as being carious. He found that ground sections were helpful in identifying such areas but that microscopic examinations did not always permit positive distinction between incipient caries pigmentation and apparently normal enamel discoloration. Certain of his photomicrographs showed gram-positive spherical microorganisms in the enamel matrix and in dentin. The research of Keyes[1] demonstrated caries in the hamster teeth by several means and utilized a large number of animals. Since this investigation technically was of an encompassing nature, it dealt only in a cursory way with the microscopic phase of the disease from the standpoint of decalcified material.

The experiment to be reported was devised to study, in minute and precise detail, the relationship the organic component of the enamel bears to the histopathology of caries, and the effect upon it of bacterial invasion. Because of the close attention which must be paid to the many factors involved in the preparation of this kind of material and the making of a serial study for microscopic examination, the molar teeth of 3 laboratory animals are reported in this study.

MATERIALS AND METHODS

The teeth studied were from normal laboratory animals. They will be designated as *A*, *B*, and *C*. They were of comparable age and their diets consisted essentially of fresh greens, oats, fox chow, and sunflower seeds. The mandibular and maxillary teeth including the alveolar process were removed from the jaws and placed immediately in 10 per cent neutral formol. A gross examination of all the teeth was made with the dissecting microscope at this

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time. Their characteristics are described under the observations of the individual teeth. Detail of the morphology of the hamster teeth is discussed in some length by Keyes and Dale.[2]

Following a 24-hour period of fixation, the molars of the 3 animals were dissected from their bony sockets and prepared for histological study. The grooves and fossae in the teeth of 1 of the animals were probed for softened areas in the enamel and dentin and, therefore, could not be used for histological study.

The remaining teeth of the 2 animals were dehydrated with 70 per cent alcohol and then decalcified for 5 hours in 80 per cent alcohol and 5 per cent nitric acid. This method of decalcification seems to yield a greater amount of organic matrix with less distortion of the tissue than other methods which have been employed in the laboratory. The dehydration was continued in 80 per cent alcohol, 90 per cent, 95 per cent, and in absolute alcohol. The final dehydration was accomplished by alcohol and ether. The material was then infiltrated for 24-hour periods each in 10 per cent nitrocellulose and 25 per cent nitrocellulose. The teeth were then embedded in 25 per cent nitrocellulose. The embedded teeth were serially sectioned on the microtome to an approximate thickness of 5 μ. The tissue sections were freed from the nitrocellulose by immersion in eugenol. The staining procedures included Gram-Weigert, MacCallum-Gram and Masson-Trichrome. Occasional sections were stained with silver nitrate after Von Kossa's method to determine whether any mineral remained in the sectioned material. In our studies, as in Keyes', a heavy organic cuticle, homogeneous in nature, covered the enamel of the anatomical crown, and was retained in the histological sections.

OBSERVATIONS

Animal *A*, female, was sacrificed at 18 months. The animal had a full complement of teeth. The low-power examination, at 14 to 20 diameters magnification, showed the teeth to be generally discolored. There were no open carious lesions. The buccal and lingual pits and occlusal fossae of the maxillary teeth appeared as darkly stained areas which contained debris and particles of food. The mandibular teeth were worn down practically to the cervical line. The maxillary molars were worn less than the mandibular molars.

The buccal and lingual pits and the occlusal fossae in the teeth of this animal were probed for softened areas. The dark plaque-like material in the pits and fossae was removed, exposing an apparently sound, but stained enamel surface. Since the surface of the pits and fossae had been disturbed by mechanical probing, no further work was done on the teeth of this animal.

Animal *B*, female, was also sacrificed at 18 months. A gross examination of all of the surfaces of the teeth, by the dissecting microscope, showed the teeth to have numerous deeply stained areas on the enamel surfaces. There were no visible cavities in the enamel. One of the maxillary first molars was selected for preparation and histological examination. The tooth was sectioned in a mesio-distal direction and 54 serial sections were made from this specimen.

The microscopic examination of the serial sections showed fragmentary remnants of organic matrix. Plaques were present in the occlusal pits and sulci. Deeply stained parts of the organic cuticle were retained in the gingival sulcus. This cuticle was heavily infiltrated with microorganisms. One of the occlusal fossae contained a plaque which was so heavily stained that it was impossible to definitely establish the relationship of the organisms within the plaque (Fig. 1). It was possible only to trace the organic cuticle to the pe-

Fig. 1.

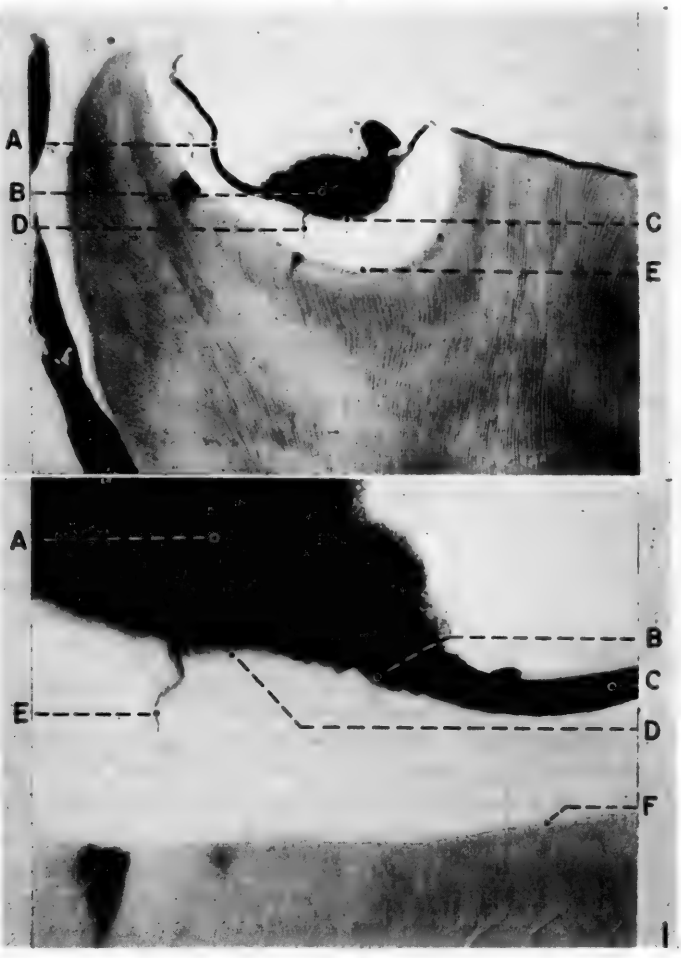

Fig. 2.

Fig. 1.—Animal *B*, maxillary 1st molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation. Gram-Weigert, ×200; illustrating a section of an occlusal sulcus containing the plaque, organic cuticle, and enamel matrix. *A*, organic cuticle; *B*, plaque; *C*, remnants of organic matrix; *D*, lamella; *E*, dentino-enamel junction.

Fig. 2.—Animal *B*, maxillary 1st molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation. Gram-Weigert ×1000; illustrating the relationship of the plaque to the organic cuticle and lamella. *A*, plaque; *B*, cuticle lost, exposing ends of organic rods; *C*, organic cuticle; *D*, eroded ends of organic rods; *E*, stained organic tract, or lamella; *F*, dentino-enamel junction.

riphery of the plaque where it became progressively more ragged and finally could not be traced beneath the plaque (Fig. 2). In the depth of the fossae the ends of the rods of the organic matrix formed an irregular surface. A few of them appeared eroded and projected beyond the others. In these sections, where the ends of the rods were exposed, the organic cuticle was entirely lost. In certain areas beneath the plaque tracts of enamel matrix stained more heavily. In some sections these tracts extended toward the dentino-enamel junction and resembled the descriptions of lamellae (Fig. 2 *E*). No organisms could be found in these areas.

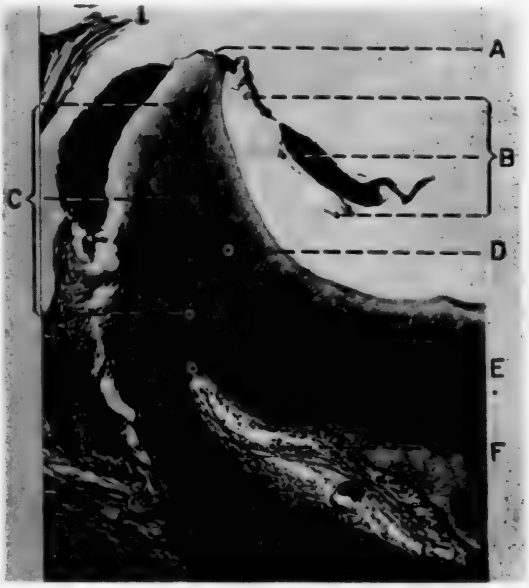

Fig. 3.—Animal *B*, maxillary 1st molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation. MacCallum-Gram, ×150; illustrating an over-all picture of the plaque and presence of microorganisms in the dentin. *A*, plaque; *B*, remnants of enamel matrix; *C*, bacteria present in dentin; *D*, dentin; *E*, predentin; *F*, pulp.

A plaque was retained over one of the cusps from which the enamel had been worn away by attrition, exposing the dentin (Fig. 3). The plaque contained numerous organisms and stained heavily. Several of the serial sections showed the presence of cocci in the dentinal tubules (Fig. 4 *B*). These organisms were observed along the lateral walls of the dentinal tubules. In several of the tubules the organisms had penetrated as far as the pre-dentin where a number of them seemed to form a bacterial focus (Fig. 4 *C*).

Animal *C*, female, was sacrificed at approximately 18 months. The gross examination with the dissecting microscope showed the teeth to be discolored with deeply stained areas in the pits and sulci. The enamel at the apices of the cusps was worn away through attrition. There were no gross cavities in the enamel. Four of the mandibular molars were prepared for histological study. Three of these were sectioned in the mesio-distal direction, while the

Fig. 4.

For legend see opposite page

fourth was sectioned bucco-lingually. The teeth will be referred to as molars 1, 2, 3, and 4. Molars 1 and 2 were imbedded in the same block and 61 serial sections showed fragments of enamel matrix in the region of the occlusal pits

Fig. 5.

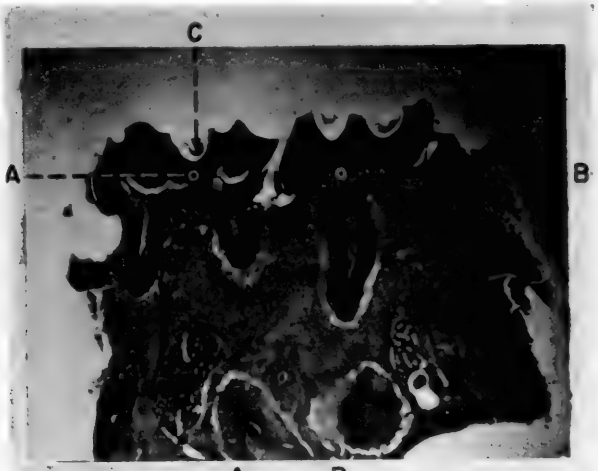

Fig. 6.

Fig. 5.—Animal *C*, molars 1 and 2, mandibular 1st and 2nd molars, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation, Gram-Weigert, ×25; illustrating an over-all picture of the occlusal sulci and plaques. *A*, 1st mandibular molar (molar 1); *B*, 2nd mandibular molar (molar 2); *C*, occlusal plaque in 1st mandibular molar.

Fig. 6.—Animal *C*, molar 2, mandibular 2nd molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation, MacCallum-Gram, ×50; illustrating the occlusal plaques, organic cuticles and enamel matrix. *A*, mesial sulcus showing organic plaque, cuticle and relationship to enamel matrix; *B*, distal sulcus showing plaque and cuticle; *C*, oral epithelium; *D*, gingival sulcus.

Fig. 4.—Animal *B*, maxillary 1st molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation. MacCallum-Gram, ×1000; illustrating the presence of bacteria in the dentin. Organisms along inner walls of tubules extending from the apex of the cusp toward the pulp, and a bacterial focus near predentin. *A*, plaque; *B*, bacteria in dentinal tubules; *C*, bacterial focus at predentin.

and fossae (Fig. 5). The enamel matrix that was preserved had the usual rod matrix formation. The greater part of the retained matrix was in good relation to the dentino-enamel junction.

Animal *C*, Molar 1 (mandibular first molar), Fig. 5 *A*. An occlusal plaque was retained which was heavily contaminated with microorganisms. Because of the dense staining of the plaque it was not possible to make a detailed examination of its contents. There were no organisms in the enamel matrix under the plaque.

Fig. 7.

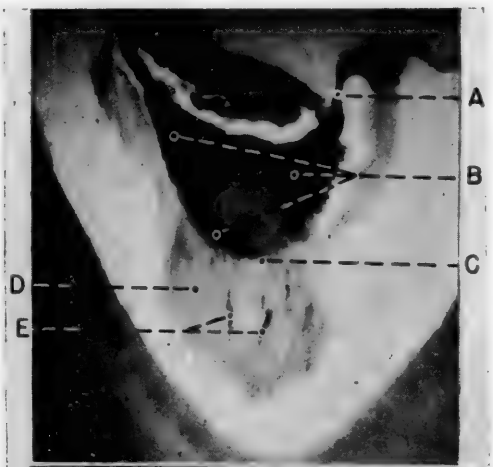

Fig. 8.

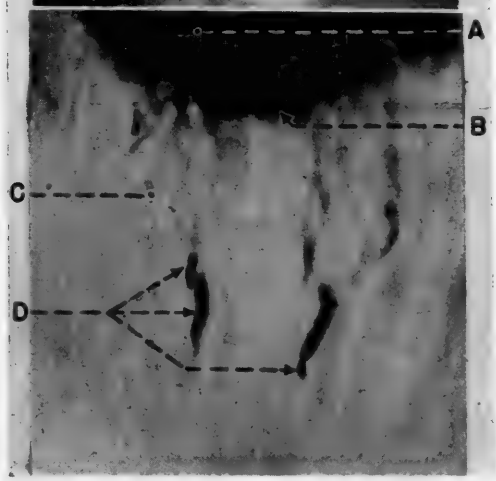

Fig. 7.—Animal *C*, molar 2, mesial sulcus, mandibular 2nd molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation, Gram-Weigert ×300; illustrating the plaque and enamel matrix in the mesial sulcus and the presence of microorganisms in the enamel. *A*, organic cuticle; *B*, plaque; *C*, depth of sulcus; *D*, enamel matrix; *E*. microorganisms in enamel matrix.

Fig. 8.—Animal *C*, molar 2, mandibular 2nd molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation, Gram-Weigert ×1000; illustrating invasion of organisms in enamel matrix (in the mesial sulcus). *A*, plaque; *B*, eroded ends of organic rods and coccoidal organisms in relationship to disintegrating enamel surface; *C*, enamel matrix; *D*, microorganisms in enamel.

Animal *C*. In Molar 2 (mandibular second molar) 2 occlusal plaques were retained in the occlusal sulci (Fig. 6 *A* and *B*).

Mesial Sulcus of Molar 2.—The plaque in the sulcus between the first and second cusps was well preserved and the relationship between the plaque and

Fig. 9.

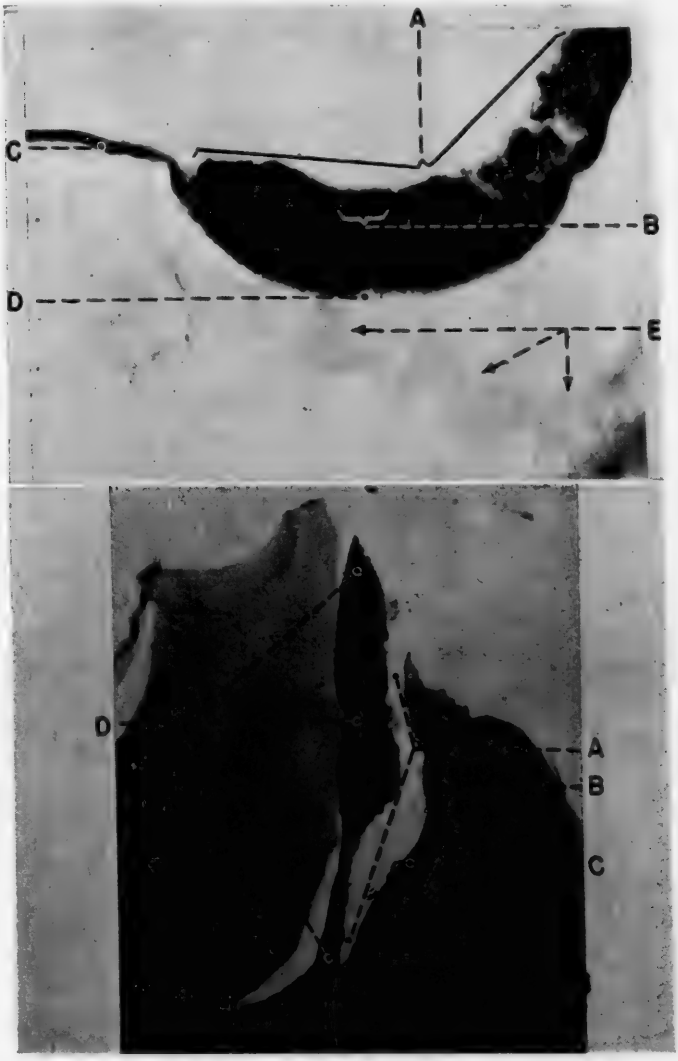

Fig. 10.

Fig. 9.—Animal *C*, molar 2, distal sulcus, mandibular 2nd molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation, Gram-Weigert, ×800; illustrating the plaque formation in the distal occlusal sulcus. *A*, plaque in distal occlusal sulcus; *B*, coccoidal organisms; *C*, organic cuticle; *D*, depth of sulcus showing disintegrated cuticle; *E*, remnants of enamel matrix.

Fig. 10.—Animal *C*, molar 2, mandibular 2nd molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation, Gram-Weigert, ×180; illustrating the gingival sulcus on the distal surface of the second molar. *A*, gingival sulcus; *B*, oral epithelium; *C*, epithelium lining the gingival sulcus; *D*, organic cuticle and plaque.

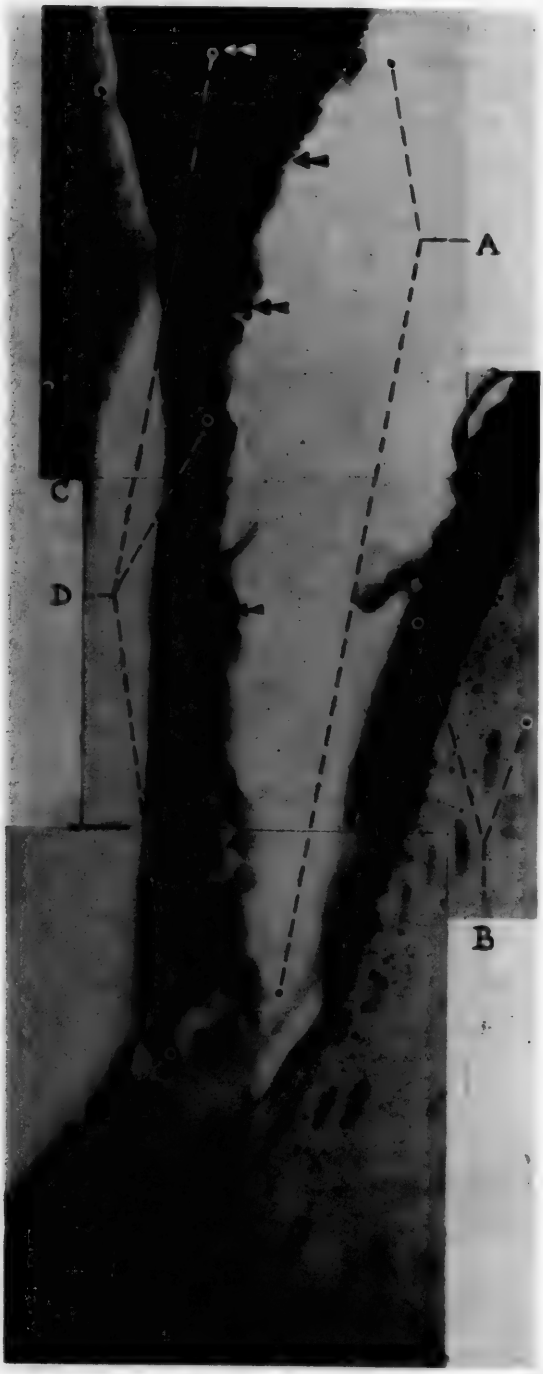

Fig. 11.

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enamel matrix was excellent. The plaque seemed to be amorphous and took the eosin of the Gram-Weigert stain. The microorganisms were predominately cocci and gram-positive. They were largely confined to the periphery of the plaque.

The organic cuticle was intact on either side of the enamel walls of the sulcus but could not be traced under the main body of the plaque (Fig. 7 *A*). The ends of the enamel rods in the depth of the sulci appeared ragged and uneven (Fig. 8). Several colonies of coccoidal organisms were seen within the enamel matrix just below the surface. Isolated groups of coccoidal forms, which seemed to occupy the core of the matrix rod, could be determined deep within the matrix (Fig. 8 *D*). There was evidence in the serial sections that these isolated colonies were connected with the more superficial caries areas near the surface. The matrix surounding the deep colonies stained, histologically, more intensely.

Distal Sulcus of Molar 2.—The plaque in the sulcus between the second and third cusps stained less heavily than the one previously described but was similar in the overall appearance (Fig. 6 *B*, Fig. 9). There were fewer organisms which seemed to be predominately coccoidal in appearance. The body of the plaque was clearly discernible and accepted the eosin of the Gram-Weigert stain. The organic cuticle showed a clear-cut picture of disintegration at the depth of the sulcus (Fig. 9 *D*). Coccoidal-appearing organisms could be determined within the eroded ends of the partially disintegrated cuticle.

The distal surface of the second molar had a well-defined organic cuticle which extended from the bottom of the gingival sulcus to almost the tip of the cusp (Fig. 10). This cuticle was formed of remnants of the reduced enamel epithelium and pyknotic epithelial cells. Since the enamel matrix was lost in this area during the preparation of the specimen, the cuticle had fallen in toward the dentin surface but remained fairly well intact (Fig. 10 *D*). The cuticle toward the occlusal surface was packed with microorganisms, while that portion of the cuticle near the bottom of the sulcus contained only a few organisms (Fig. 11). In the more superficial areas of the cuticle the organisms were predominately threadlike forms while near to the enamel surface they were coccoidal in shape (Fig. 11).

Animal *C*, Molar 3 (mandibular first molar) sectioned in a mesio-distal direction. Forty-one serial sections were made from the tooth, but were not suitable for photomicrographic reproduction. Only isolated remnants of the enamel matrix were recovered, the majority being lost by the demineralization process. Remnants of the organic cuticle were saved, some of which retained their continuity with the matrix and the plaque. The apices of the cusps were abraded to the extent that the enamel was entirely gone.

Microscopic plaques on the occlusal surface were observed in many of the serial sections. These were found in the deepest part of the occlusal sulci and

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Fig. 11.—Animal *C*, molar 2, mandibular 2nd molar, hamster, 18 months, decalcified mesio-distal section, 5 μ neutral formol fixation. Gram-Weigert, ×1000; illustrating the gingival sulcus on the distal surface of the second molar. *A*, gingival sulcus; *B*, sulcus epithelium; *C*, dentin; *D*, organic cuticle and plaque.
Arrows indicate microorganisms.

grooves. In some sections a fair relationship of the plaque, organic cuticle, and a continuous remnant of the enamel matrix approximating the cuticle could be determined. The plaque presented the usual amorphous background which contained a multitude of organisms. The coccoidal forms seemed to predominate. In one section the plaque was covered with debris which was relatively free of organisms. There was no deep invasion of the matrix in this specimen and no dentin involvement could be determined. Several heavier staining lamellae were observed extending between the cuticle of the enamel surface and the dentino-enamel junction. These lamellae, although they stained histologically more intensely than the surrounding matrix, did not contain organisms.

The gingival sulcus contained remnants of the organic cuticle and a definite plaque which closely resembled that found in the occlusal surface. Several of the sections were carefully stained with crystal violet for microorganisms and yielded an excellent picture with threadlike microorganisms predominating in colony-like formation. The coccoidal variations were in the minority and were located approximating the enamel surface. In many instances granules could be determined at the ends of the threads, and these were located in the plaque nearest the tooth surface.

Animal *C*, Molar 4 (mandibular second molar) sectioned in a bucco-lingual direction, from which 91 serial sections were made. The enamel of the apices of the cusps was worn away through attrition, and, therefore, no enamel matrix could be recovered from these areas. The microscopic study of this tooth was largely confined to 2 occlusal fossae which will be designated as the mesial and distal.

Mesial Fossa of Molar 4.—The plaque lying in the mesial fossa of the occlusal surface was extremely dense and contained a multitude of organisms (Fig. 12). The plaque and organic cuticle were so deeply stained that the precise relationship of plaque to cuticle in some of the sections could not be determined. In this section the plaque was in direct continuity with a lamella which extended between the enamel surface and the dentino-enamel junction (Fig. 12 *F*).

In the other serial sections, taken laterally from the central part of the fossa, lamellae were observed to extend from the dentino-enamel junction to become continuous with the surface cuticle (Fig. 13 *A*). Both the lamellae and the cuticle stained more intensely than the surrounding enamel matrix. Certain of the tracts or lamellae at the enamel surface were somewhat funnel-shaped and crowded with coccoidal microorganisms (Fig. 13 *C*). Other lamellae had a similar appearance (Fig. 14 *C*). Occasional coccoidal forms were seen to occupy the tract toward the dentino-enamel junction (Fig. 14 *B*).

An examination of the dentin lying immediately beneath another lamella showed that the microorganisms had penetrated along several dentinal tubules as far as the predentin of the pulp (Fig. 15). In some of the tubules there seemed to be a lateral spreading of the bacteria in the dentin (Fig. 16 *B*). There was apparently no pulp reaction resulting from the close approximity of the

organisms at this time. The microorganisms seen in the dentin were morphologically the same as those observed along the surface of the organic cuticle and within lamellae.

Fig. 12.

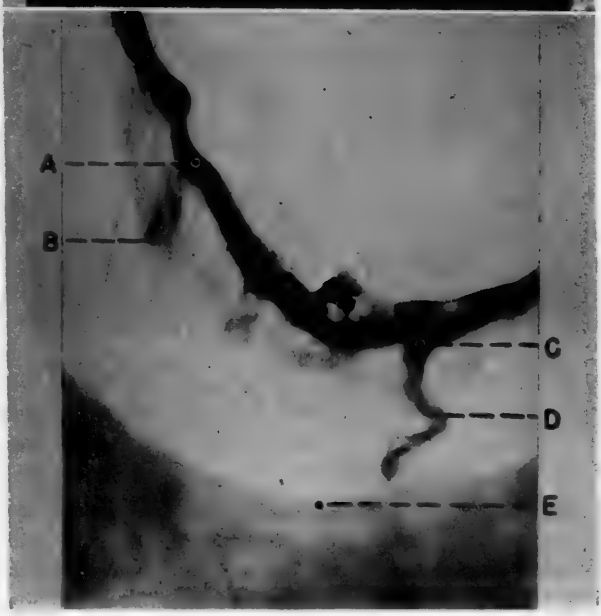

Fig. 13.

Fig. 12.—Animal *C*, molar 4, mesial fossa, mandibular 2nd molar, hamster, 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation, Gram-Weigert, ×300; illustrating the plaque in the mesial fossa of the occlusal surface. *A*, organic cuticle; *B*, enamel matrix; *C*, dentino-enamel junction; *D*, dentin; *E*, plaque; *F*, lamella.

Fig. 13.—Animal *C*, molar 4, mandibular 2nd molar, hamster, 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation, MacCallum-Gram, ×1000; illustrating the lamella extending between the dentino-enamel junction and the surface cuticle in the lateral section of the mesial fossa. *A*, organic cuticle; *B*, remnants of enamel matrix; *C*, organisms in lamella; *D*, lamella; *E*, dentino-enamel junction.

Distal Fossa of Molar 4.—A dense bacterial plaque occupied the deepest part of the pit. A differentiation between the organic cuticle and the inner surface of the plaque could not be made in the sections taken from the center of the plaque. In the more lateral sections the cuticle could be determined in relation to the organic rods of the enamel matrix (Fig. 17). A definite break in the cuticle was evident and bacteria had penetrated and spread laterally into the enamel (Fig. 18).

The serial sections showed that the organisms may have gained an entrance through the disintegrated organic cuticle, establishing a large focus of bacteria in the enamel approximately midway between the dentino-enamel junction and the enamel surface (Fig. 18 *D*). The focus had spread laterally so as to undermine the intact surface and contained many coccoidal forms (Fig. 19 *B*). The bacteria were confined to this circumscribed area and no involvement of the dentin could be found in any of the serial sections.

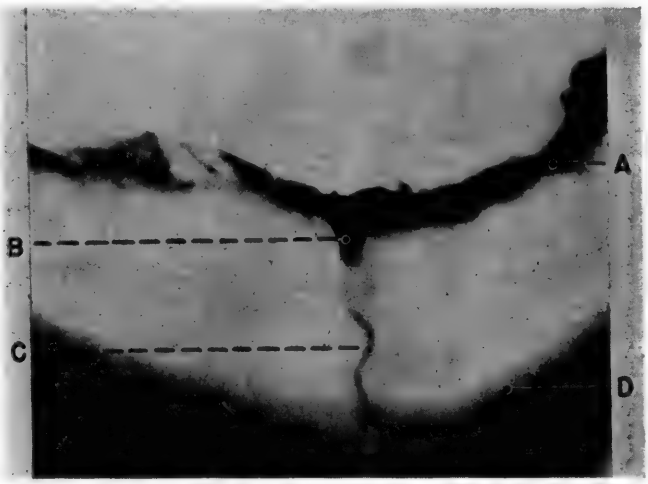

Fig. 14.—Animal *C*. Molar No. 4. Mandibular 2nd molar, hamster, 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation. MacCallum Gram. ×1000. Illustrating the organic cuticle and microorganisms present in the lamella in a lateral section of the mesial fossa. *A*, organic cuticle; *B*, coccoidal organisms in the lamella; *C*, lamella; *D*, dentino-enamel junction.

DISCUSSION

The purpose of this investigation was to study by histological methods the relationship the organic component of the enamel bears to the histopathology of early enamel caries. Although relatively few teeth were used in the investigation, an attempt was made to study them by following out precise methods of preparation and staining and serial sectioning. From these serial sections it seemed possible to present a fairly accurate histological concept of the early caries lesion of the organic cuticle and lamellae in the enamel of the Syrian hamster.

The fossae and sulci in the teeth of one animal were studied for orientation, under the low power dissecting microscope. The debris of the plaque was re-

moved, revealing a brown-stained area of enamel. These areas were probed and the enamel surface seemed to be intact and sound. No gross lesions or cavitation of the enamel were observed in any of the animals studied, although cavitation in similar areas has been demonstrated by other investigators.[1, 3]

This histological study showed 2 types of plaques: those occurring in the fossae and sulci of the occlusal surfaces and those in the gingival sulci.

The microscopic plaques found in the occlusal surfaces appeared to be quite dense and the histological stains revealed many organisms. These organisms were predominately coccoidal in form and gram-positive, although thread forms were observed in many of the sections. When the matrix or the background of the plaques could be observed, it appeared to be of an amorphous structure and accepted the acid stain.

In the gingival sulci the matrices of the plaques were formed of remnants of the enamel organ and pyknotic epithelial cells. These plaques were heavily infiltrated with both threadlike organisms and coccoidal forms. The thread forms occupied the more superficial areas, whereas the coccoidal forms were in greater abundance in the deeper structure of the plaques.

The anatomical crown of the molar was covered with a heavy organic cuticle. In the histological sections this cuticle was homogeneous and stained darker than did the enamel matrix.

With the onset of caries this organic cuticle became ragged beneath the plaque. In the more advanced lesion it was completely lost exposing the ends of the organic enamel rods. When the ends of the rods were exposed, they presented an eroded appearance. As the lesion advanced, coccoidal organisms were observed deep within the enamel matrix.

In occlusal fossae and sulci of the teeth, darker staining organic tracts or lamellae were observed extending between the enamel surface and the dentino-enamel junction. These organic tracts appeared to be continuous with the surface cuticle. In many of the serial sections bacteria could be determined along these tracts to the dentino-enamel junction where they were seen to penetrate the dentin.

In serial sections of 2 specimens organisms had definitely penetrated into the dentin. These organisms appeared as cocci and seemed to be located in the lateral walls of the tubules. Only a few tubules were involved but the bacteria had penetrated as far as the predentin where they established a small focus. It was not possible to determine clearly whether there was a pulp response as the result of the focus.

From the above histological picture it would seem that the organic cuticle directly beneath the bacterial plaque is the first to undergo a breakdown in the caries process. With its destruction the ends of the rods and interrod intervals were opened up and the rods appeared to undergo a breakdown. Following the destruction of the cuticle and the opening of the rods, organisms were observed along specific organic tracts. These paralleled the direction of the enamel rods and appeared to be lamellae. These tracts were continuous with the surface cuticle and could not be determined by gross visual means.

Fig. 15.

Fig. 16.

Fig. 15.—Animal *C*, molar 4, mesial fossa, mandibular 2nd molar, hamster, 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation, Gram-Weigert, ×200; illustrating the microorganisms in the dentin (under the area of the mesial fossa). *A*, dentin; *B*, microorganisms; *C*, predentin; *D*, pulp.

Fig. 16.—Animal *C*, molar 4, mandibular 2nd molar, hamster, 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation, MacCallum-Gram, ×1000; illustrating the focus of organisms in the dentin. *A*, dentin, *B*, lateral spreading of bacteria in dentinal tubules.

Fig. 17.

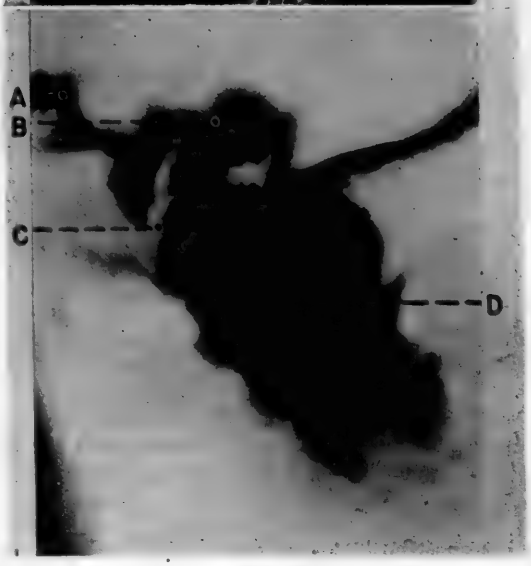

Fig. 18.

Fig. 17.—Animal *C*, molar 4, distal occlusal fossa, mandibular 2nd molar, hamster, 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation, Gram-Weigert, ×175; illustrating the bacterial invasion in the enamel matrix. *A*, bacterial invasion into enamel; *B*, bacterial focus; *C*, organic cuticle; *D*, enamel matrix; *E*, dentino-enamel junction.

Fig. 18.—Animal *C*, molar 4, distal fossa, mandibular 2nd molar, hamster 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation, MacCallum-Gram, ×1000; illustrating the break in the cuticle and spread of microorganisms in the enamel. *A*, intact organic cuticle; *B*, disintegrated organic cuticle; *C*, enamel matrix; *D*, coccoidal organisms in the enamel.

The plaque of the gingival sulcus, which was principally composed of keratinized epithelial cells and remnants of the enamel organ, was heavily infiltrated with microorganisms. This observation may throw some additional light on the occurrence of caries lesions in the hamster, as observed by King.[4] He reported that the lesions arose in the cementum and entered into the enamel by the way of the dentin.

In the dentin organisms were observed to penetrate directly from the surface, apparently extending along the lateral walls of the tubules to the predentin. This occurrence resembled that observed by Keyes[1] in the hamster and Frisbie, Nuckolls and Saunders[5] in the human being.

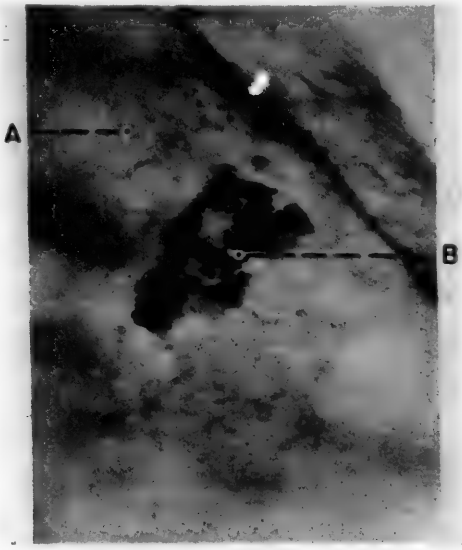

Fig. 19.—Animal *C*, Molar 4, distal fossa, mandibular 2nd molar, hamster, 18 months, decalcified bucco-lingual section, 5 μ neutral formol fixation, Gram-Weigert, ×1000; illustrating the microorganisms in the enamel matrix. *A*, enamel matrix; *B*, coccoidal organisms.

The organisms of the plaques were predominately cocci and thread forms, while those seen in the organic tracts of the enamel and the dentin were cocci. The studies of Hurst, Frisbie, Nuckolls and Marshall[6] on actinomycetes recovered from the tooth surfaces of both hamsters and human beings indicate that these organisms are highly pleomorphic and may appear in culture both as cocci and threadlike structures. These studies have shown that pure strains of actinomycetes will produce microscopic carieslike lesions in vitro when unerupted hamster molars are infected with the organisms. When they invade these teeth, they sometimes assume the coccoidal form.

While a histological study portrays only an alteration in structure, it seems reasonable to assume that the very early phases of caries in the hamster, especially of the enamel, are first concerned with an enzymatic breakdown of the organic structure. This, in turn, may liberate organic acid capable of a

demineralization of the inorganic constituents as suggested by Atkinson and Matthews.[7] This mechanism could provide for the extension of the disease which ultimately results in cavitation.

SUMMARY

A histological study of the organic matrix of the enamel and dentin of a limited number of the teeth of the Syrian hamster indicates that the early phases of the disease of caries may be associated with the early breakdown of the organic component of the structure. In this respect caries in the hamster seems to be similar to that in the human being. This mechanism includes the presence of organisms in the plaque, the breakdown of the organic cuticle followed by the invasion of coccoidal organisms into specific tracts in the enamel and along the lateral walls of the dentinal tubules. This early phase of caries of the lamellae precedes the formation of open cavities, is microscopic in nature and can be observed only by histological methods.

It is postulated that the early extension of the disease process is concerned with an enzymatic breakdown of the organic matrix together with the presence of an acid which would produce a demineralization of the apatite lattice of the enamel.

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ARTIFICIAL DENTAL CARIES

Preliminary Report

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MANY investigators[1-10] have demonstrated in vitro carieslike dental changes in extracted human teeth exposed to media containing bacteria. Some reports[8-10] show the preservation of surface contour during the earlier stages of artificial caries. The essential feature of the experiment reported here is the in vitro production of a change in the tooth having a number of characteristics indistinguishable from those observed in early clinical caries. These characteristics are:

1. Normal surface contour and gloss;
2. Pigmentation of the affected areas;
3. Radiographic rarefaction extending through the enamel;
4. Softening of the enamel (as determined by pressure with a dental explorer);
5. Spreading of the process at the dentinoenamel junction.

EXPERIMENTAL

Three premolar teeth (Fig. 1, *A*, *B*, and *C*) from different patients were used. *A* is from a 35-year-old woman with rampant caries; *B* is from a 50-year-old man showing immunity to caries; and *C* (showing characteristic mottling) is from a 36-year-old woman who was born and reared in a region of endemic fluorosis and exhibits few carious lesions.

The teeth were mounted together in one block of modeling compound (Fig. 1) and then covered with sticky wax except for small areas on the proximal and buccal surfaces.

One cubic centimeter of fresh unstimulated saliva, from a 35-year-old man (D.M.F. index $\frac{18}{32}$) exhibiting apparently no active carious lesions, was added to a mixture of 100 c.c. of Berewer's medium supplemented with 2 Gm. of bacto-dextrose.

The block containing the three teeth was immersed in the mixture and incubated at 37° C. After 24 hours of incubation pH readings ranged from 4.3 to 5.0. The mixture was renewed weekly and the experiment was continued in this manner for six months.

Fig. 2 shows an x-ray film and photographs of the teeth at the conclusion of the experiment. Radiographic, typical carieslike penetration throughout the enamel is seen in teeth *A* and *B*. Tooth *C* (mottled enamel) shows shallow and less typical rarefaction. The photographs show that surface continuity and surface gloss are preserved except in the buccal areas which were pierced with

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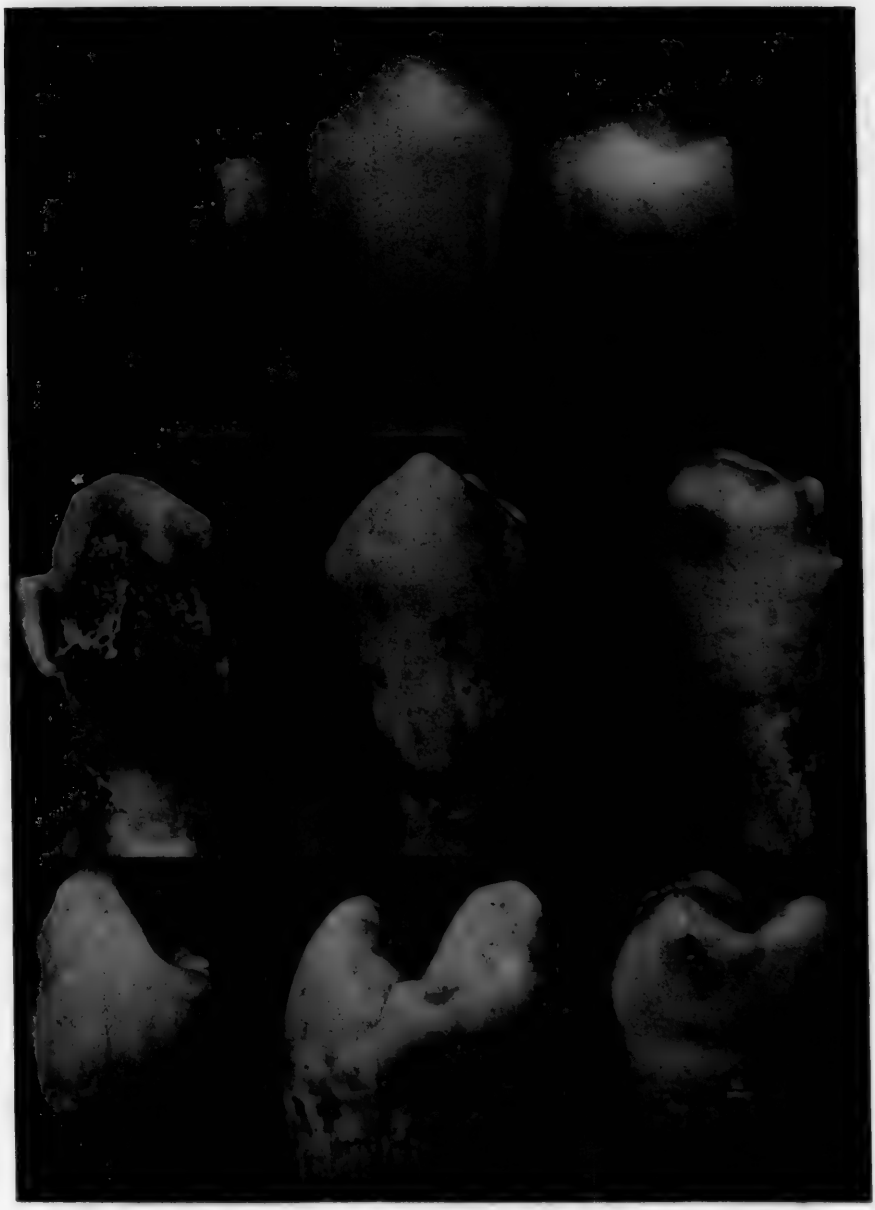

Fig. 1.—Top row shows x-ray negative of teeth at start of experiment. Middle and bottom rows are photographs of teeth at start of experiment. Column *A* shows views of tooth from patient with rampant caries; Column *B* shows views of tooth from patient immune to caries; Column *C* shows views of tooth from patient with mottled enamel.

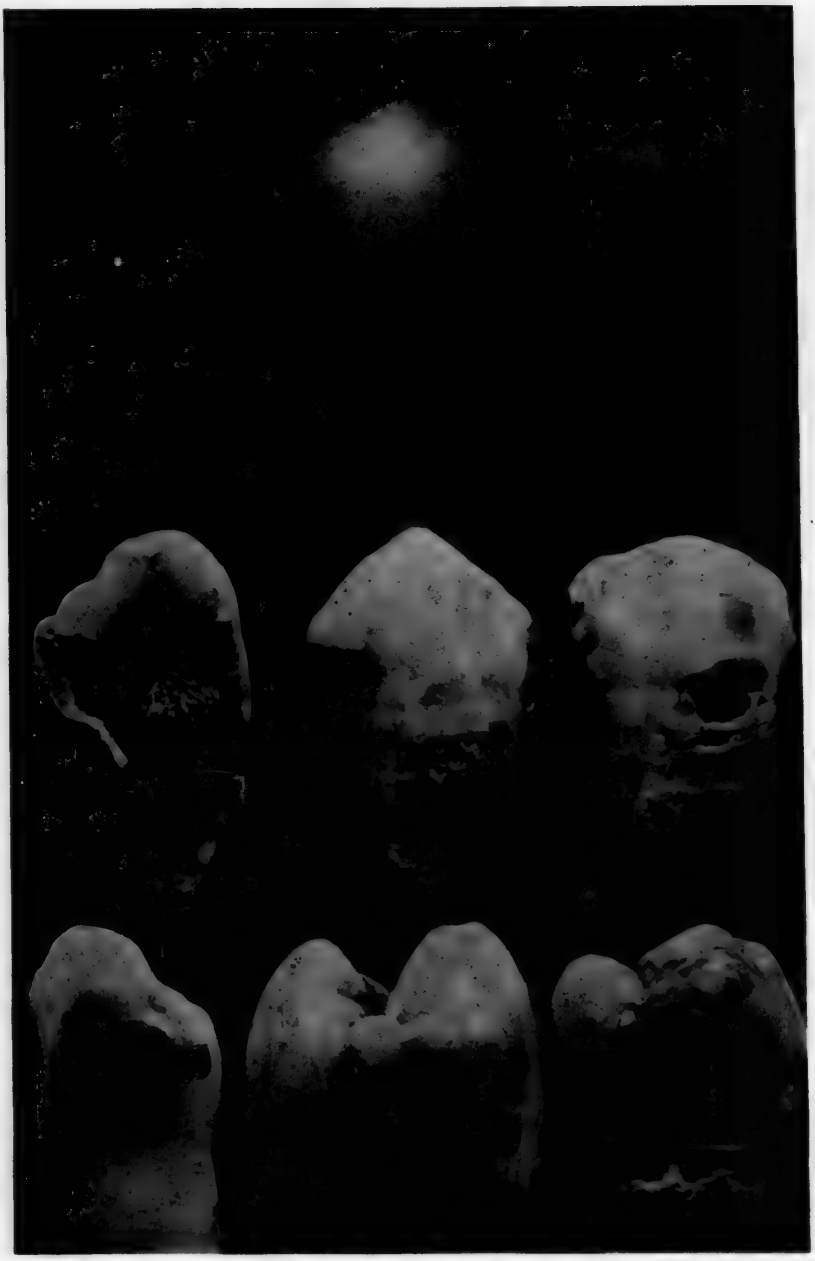

Fig. 2.—Top row shows x-ray negative of teeth at the conclusion of the experiment. (Note rarefaction on approximal surfaces.) Bottom row shows pigmentation, intact surface contour and gloss. Middle row shows buccal "lesions" in teeth *B* and *C*. The buccal region in tooth *C* was pierced with an explorer to show softened enamel. The buccal area in tooth *C* was excavated to show softened dentin and spreading at the dentinoenamel junction.

a dental explorer to denote softening of the tooth substance. The color of the affected areas is grayish brown. The excavation of the buccal region of tooth *C* showed the dentin to be soft, leathery, and discolored (dark brown). The softened dentin spread at the dentinoenamel junction undermining the unaffected enamel.

SUMMARY

Phenomena indistinguishable in pattern from clinical caries have been produced in vitro in three human teeth.

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HISTOLOGICAL FINDINGS IN THE SALIVARY GLANDS OF THE RAT FOLLOWING SODIUM FLUORIDE ADMINISTRATION

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THE influence of fluorine upon the living body has been the subject of much study. DeEds,[1] Greenword,[2] Roholm,[3, 4] and Sollmann,[5] have reviewed adequately the occurrence, distribution, and major chemical characteristics of the element and the salts of which it forms a part.

Sodium fluoride is sharply contrasted to most other salts of hydrofluoric acid because of its marked solubility in water[6] and it is characterized by almost complete dissociation.[7] Furthermore, a direct relationship between fluoride solubility and toxicity has been confirmed by recent studies.[8] When administered in solution, even the less soluble fluoride salts are readily absorbed. It follows logically that, to a certain extent, absorption is dependent upon aqueous solubility.[9]

Special significance must be attached to the biochemical characteristics of the fluorides. The majority of investigators conclude that absorbed fluorides act primarily upon the enzymatic systems of the body.[5, 10, 11] Some enzymes, such as lipase[12] and the esterases, are markedly inhibited, while salivary amylase and the proteolytic enzymes are but slightly sensitive.[5, 13] The reviews of McClure[14] and Sollmann,[5] and the studies of Handler,[15] and Kaplan and Greenberg[16] stress the powerful inhibitory effects of fluorides upon carbohydrate metabolism. Phosphorylating and dephosphorylating mechanisms concerned in the breakdown of glycogen and in the oxidation of glucose are specifically inhibited.[8, 17, 18] The effect is due, first, to a depression of the enzyme enolase that converts 2-phosphoglyceric acid into 2-phosphopyruvic acid.[5] A second inhibitory action, involving the coenzyme adenosine triphosphate occurs at the same time.[15, 17, 19]

The enzyme systems controlling tissue respiration do not escape damage. Even moderate doses of fluoride have been noted to produce diminished respiration in tissues[19, 20] and a decrease in both the O_2 consumption and CO_2 production of the intact animal.[21] Borei[22] has suggested that the fluoride-sensitive link in the respiratory chain lies in the cytochrome system. The decrease in liver cytochrome oxidase of animals (chronically poisoned by fluoride), as noted by Phillips and Chang,[23] adds strength to this argument.

A definite modification of calcium and phosphorus metabolism occurs as a result of fluoride intake. Negative calcium balance[3] and decreased retention of calcium and phosphorus[2, 24] appear of consequence. The diminution of blood calcium which is characteristic of fluoride toxicosis can be assumed to stem in part from the removal of fluoride as insoluble CaF_2.[8, 25] Calcium injection gives an antagonistic effect in such instances through defluoridation.[5] However, CaF_2 precipitation does not provide the entire reason for the observed changes, nor explain fluoride toxicity. The fact that calcium injection during fluoride poison-

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ing will not correct the calcium deficiency[5] points to a concomitant disturbance of enzyme function.

Present knowledge of the mechanisms involved in fluoride intake storage and excretion is quite incomplete. Machle and his co-workers emphasize, first, that when intake plus absorption does not exceed a certain low level there is an equilibrium between intake and output; second, that skeletal storage of fluoride is superimposed upon urinary excretion at higher chronic levels of intake; third, that the amounts stored and excreted vary directly with the amounts absorbed.[9, 26]

Although detailed clinical and epidemiological reports of the dental effects of fluoride intake are already available,[27-30] information as to the sites of action and nondental effects of fluorides is incomplete. In particular, histologic studies of viscera after acute fluoride intoxication are sparse. Roholm's review[3] describes "islands" of incipient necrosis in the liver, as well as renal hemorrhage, inflammation and tubular degeneration, after acute toxicosis. The profuse salivation almost synonymous with very acute toxemia[3, 15] is well known. Despite this, significant histological change in the salivary glands has not been reported[3, 31]

MATERIAL AND PROCEDURE

Two groups of rats of the Long-Evans strain were studied, each group consisting of ten experimental and five control animals. The two groups were designated as "acute" and "chronic," based on the duration of the experimental period and amount of sodium fluoride administered. Rats of the acute group were 49 days old at the onset of injections, those of the chronic group 52 days old. All rats received a modified McCollum's diet. Animals of both groups were weighed at frequent intervals throughout the experiment.

The intraperitoneal route of injection was selected because it permits an efficient absorption of salts from the peritoneum and also produces less local tissue destruction than does injection elsewhere. Feeding of the agent would not have permitted accurate determination of the dosage.

A preliminary experiment was conducted to determine tolerance of NaF and the dosage levels. The dosage employed by Schour and Smith[32] (0.3 c.c. of 2.5 per cent aqueous NaF) was lethal in these younger animals. However, when virtually the same total amount of salt (6.9 mg. vs. Schour's 7.5 mg.) was administered in isotonic solution, it was tolerated. The initial dose for the acute group was therefore at this level, and subsequent daily (for 15 days) injections of the isotonic solution were varied in volume to maintain the rats in a state of acute toxicosis (Table I). The chronic group received injections of the isotonic solution on alternate days and in lower volumes continuing for 100 days (Table II). Controls in each group received injections of physiologically isotonic sodium chloride solution in volumes equal to those given the experimental rats. By injecting at different points in the same general area at the same intervals of time, it was possible to obviate differences in reaction which might have biased the results. Varying amounts of the solutions were administered as the experimental period advanced, in order to produce the desired toxicosis and yet maintain all possible animals alive. No attempt was made to vary the dosage of solution with changes in the animal's weight.

The autopsy of animals in each group was conducted upon the last day of injections.

Portions of the three glands were fixed in Bouin's solution. Duplicate fixation of the submaxillary and sublingual gland tissues with Zenker-formol was carried through in order to preserve more satisfactorily the granule content of the individual acinar cells. All tissues were embedded and sectioned in nitrocellulose at 7 micra thickness. Iron hematoxylin-aniline blue[33] was used to stain the sections of the parotid gland, while iron hematoxylin-aniline blue-methyl green was employed in the case of the submaxillary and sublingual glands. This latter stain permitted a far more accurate study of the mucous alveoli. A modified Mallory-azan stain[34] was also used upon sections of the submaxillary and sublingual glands.

Several unique anatomical features of the rat have a bearing upon this study and should therefore be mentioned at this point: (a) the major sublingual gland[35] is in extremely close apposition to the submaxillary, lying within the same outer connective tissue capsule; (b) the serous cells of the submaxillary gland[36] fall into two groups; those of the first group contain large highly refractive granules which are easily fixed by Zenker-formol and stain deeply with hematoxylin. These cells will be referred to hereafter in the paper as Type 1 cells. Stormont found them to lie in terminal segments of the intralobular ducts. The second group (Type 2 cells) whose secretion granules are scarcely seen in Zenker-formol fixed material, are the clear or *tropochrome* cells[37, 38] (Fig. 5 *B*). The submaxillary gland is of the pure albuminous or serous type.[39]

TABLE I

INJECTIONS OF SOLUTIONS INTO ACUTE GROUP ANIMALS (CONTROL AND F-INJECTED)

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Volume injected	0.8	0.8	1.0	1.0	1.0	1.0	1.2	1.2	1.0	1.2	1.2	1.2	1.2	1.2	1.2
Cumulative volume injected	0.8	1.6	2.6	3.6	4.6	5.6	6.8	8.0	9.0	10.2	11.4	12.6	13.8	15.0	16.2

Each control and each experimental animal received the same volume of its respective solution on a given day. Therefore, the total volume of isotonic NaCl absorbed by a control animal equaled that of the isotonic NaF absorbed by an experimental animal.

RESULTS

Gross Examination of Animals.—A total of seven fluoridized animals did not survive the entire injection periods. Total weight gains of fluoride-injected animals were lower than those of the controls on saline, but the differences in weight increase were not statistically significant (Table III). The experimental animals, as examined at autopsy, resembled the controls in gross appearance and in the macroscopic structure of their organs. Approximately one-half of the fluoride-injected animals of both groups showed skin lesions at the injection site. The classic gross effects of fluorine upon enamel substance were noted, but not in constant degree.

Microscopic Examination of Organs.—

Parotid gland:—Analysis of the parotid gland was made from separate tissue sections stained by the I.H.A.B. and modified Mallory-Azan procedures.

TABLE II

INJECTIONS OF SOLUTIONS INTO CHRONIC GROUP ANIMALS (CONTROL AND F-INJECTED)

	VOLUME OF SOLUTION INJECTED						
		F-INJECTED ANIMALS					
DAY	CONTROL ANIMALS	B45, GH16, B32, GH50, BH38	W13	B68	G51	GH17	GH66
2	.5	.5	.5	.5	.5	.5	.5
4	|	.5	|	|	|	|	|
6	|	.5	|	|	|	|	|
8	|	|	|	|	|	|	|
10	|	|	|	|	|	|	|
12	|	|	|	|	|	|	|
14	|	|	|	|	|	|	|
16	|	|	|	|	|	|	|
18	|	|	|	|	|	|	|
20	|	|	|	|	|	|	|
22	|	|	|	|	|	|	|
24	|	|	|	|	|	|	|
26	|	|	|	|	|	|	|
28	|	|	|	|	|	|	|
30	1.0	1.0	1.0	1.0	1.0	1.0	1.0
32	|	|	|	|	|	|	|
34	|	|	|	|	|	|	|
36	|	|	|	D	|	|	|
38	|	|	|		|	|	|
40	|	|	|		|	|	|
42	|	|	|		|	|	|
	|	|	|		|	|	|
44	|	|	|		|	|	|
46	|	|	|		|	|	|
48	0.6	0.6	0.6		D	D	0.6
50	0.6	0.6	0.6				D
52	0.8	0.8	0.8				
54	0.8	0.8	0.8				
56	0.8	0.8	0.8				
58	1.0	1.0	1.0				
60	|	|	1.0				
62	|	|	|				
64	|	|	|				
66	|	|	|				
68	|	|	|				
70	|	|	|				
72	0.9	0.9	0.5				
74	0.9	0.9	0.9				
76	0.9	0.9	0.9				
78	1.0	1.0	0.9				
80	1.0	1.0	0.9				
82	1.2	1.2	1.2				
84	1.2	1.2	1.2				
86	1.4	1.4	1.4				
88	1.6	1.6	|				
90	1.4	1.4	|				
92	|	|	|				
94	|	|	|				
96	|	|	1.0				
98	|	|	|				
100	|	|	|				
Total volume	45.1	45.1	43.1	10.0	16.0	16.0	16.6

All volumes are expressed in c.c.
Vertical broken line indicates the same volume on successive days.
"D" signifies that the animal died on that day.

Controls of the acute group were compared to controls of the chronic groups and the experimental tissues of both groups were contrasted. It was found that alveolar cells of acute group control animals (as compared to chronic group control animals) gave evidence of greater mitotic division (Table IV). On the other hand alveolar cell nuclei were significantly increased in size throughout the parotid glands of the older chronic group animals, control and experimental (Figs. 1 and 2). The index of significance* for the chronic group controls (as contrasted to the acute group controls) was 5.25 and for the experimental animals 5.24 (Table V).

STATISTICAL METHODS EMPLOYED

1. In the case of all data, the arithmetic mean, standard deviation and standard error of the mean were calculated.

Symbols

M = arithmetic mean of observations
Σ = "the sum of"
χ = the value of an individual observation
n = the total number of observations
d = the deviation of an individual observations from the mean
s = standard deviation
s_M = standard error of the mean

Formulae

Arithmetic mean ---------- $\frac{\Sigma \chi}{n}$

Standard deviation ---------- $\sqrt{\frac{\Sigma d^2}{n}}$

Standard error of the mean ------ $\frac{s}{\sqrt{n}}$

2. The standard errors were corrected by applying the correction factor of Pearl[42]

Correction factor ---------- $\sqrt{\frac{n}{n-1}}$

3. Statistical significance

Test of the statistical significance of the difference between two means was made as follows: If the ratio of the difference between the means to the standard deviation of the difference was equal to or greater than 3, then it was assumed that the difference between the means was due to something other than chance or random selection of animals.

$$\frac{M_1 - M_2}{\sqrt{(s_{M1})^2 + (s_{M2})^2}} \geq 3$$

4. References: Barlow,[40] Dunlap and Kurtz,[41] Pearl[42]

*The explanation of statistical methods in statistical methods employed.

TABLE III

WEIGHT CHANGES IN THE SODIUM FLUORIDE-INJECTED ANIMALS*

		WEIGHT OF THE ANIMALS IN GRAMS							
		START OF THE EXPERIMENT				END OF THE EXPERIMENT			
EXPERIMENT	GROUP	M ± s_M		s	INDEX*	M ± s_M		s	INDEX
Acute	Control	163.5	18.41	41.18	0.203	234.8	18.38	41.12	1.41
	F injected	159.7	3.7	10.47		203.0	13.08	34.64	
Chronic	Control	160.0	16.08	27.96	1.04	395.4	31.03	62.19	
	F injected	178.4	7.16	20.28		385.8	7.61	17.33	0.30

*Index = index of statistical significance (Page 716).

The second phase of parotid gland examination consisted of a comparative study of sections from experimental and control animals of the acute and chronic groups (Figs. 3 and 4). The parotid glands of fluoride-injected animals of both groups contained a greater number of ducts than did the glands of control animals, although the increase was less in the acute group. The indices of significance were 1.35 (acute group) and 2.08 (chronic group), neither of these indices being strongly positive. Mitotic division of alveolar cells was more

TABLE IV

PAROTID GLANDS: CELL MITOSIS COUNT IN ACINAR TISSUE

	CONTROL				F-INJECTED				
EXPERIMENT	ANIMAL	NO. OF MITOSES	M ± s_M	s	ANIMAL	NO. OF MITOSES	M ± s_M	s	INDEX
					BH21	13			
					BH09	36			
					G69	5			
Acute	GH00	2			GH04	10			
	B73	1			G96	100	28.16 ± 15.1	33.8	1.91
	W96	1	1.33 ± .0032	.0046	B25	5			
					W13	3			
					B45	17			
	B72	0			GH16	18			
Chronic	G57	1	.33 ± .21	.469	B32	13	15.33 ± 2.725	6.599	5.49
	G87	0			GH50	16			
					BH38	25			
Acute			1.33 ± .0032	.0046					22.7
Chronic			.33 ± .21	0.469					
Acute							28.16 ± 15.1	33.8	0.83
Chronic							15.33 ± 2.725	6.599	

The totals in columns 3 and 7 indicate the number of mitotic alveolar cells visible in ten high power fields (Ocular 10×, Objective 40×, Magnification 400) of each slide.

prevalent in experimental glands of both groups (Table IV) but was significant only for the cells of the chronic group experimental tissues (index 5.49). Similarly, the nuclei of the alveolar cells were larger in the experimental animals, especially in those (Table V) of the chronic group (index 9.74). Alveolar cell cytoplasm of the intoxicated animals showed evidence of change. Many cells did not illustrate normal uniformity of cytoplasmic staining. Clear unstained circular areas were often visible within the cells.

Five additional observations which served to distinguish experimental tissues were made during the parotid gland analysis: (1) the cell membranes of alveolar cells lost their normal clarity; (2) the separation of alveoli increased; (3) the amount of intralobular connective tissue seemed to show a corresponding increment; (4) the size of individual alveoli decreased; (5) the experimental sections stained by the modified Mallory-Azan procedure contained more prominent acidophilic granules and more granules per individual cell.

The differences between experimental and control material reported in the preceding paragraph are the least conclusive, and may not be the result of experimental conditions imposed. Nevertheless, the fact remains that they were points of interest generally noted.

Submaxillary gland:—Certain concrete differences distinguished the glandular tissue of the acute group animals from that of the older chronic group (Figs. 5 and 6). Ducts of interlobular and intralobular type were more numerous in the glands of the acute group (Table VI). An index of 2.8 suggests a probably significant difference. On the other hand, the older chronic group controls have Type 1 cell alveoli of greater number and size. The secretion granules in Type 1 cells took the acidophilic stain more deeply in sections cut from glands of the chronic group. These constant differences may stem from a modification of structure as the animal age increases.

Observations of change in the experimental submaxillary tissues lend themselves to summary (Figs. 6 and 8). There was a marked and significant increase in the number of Type 1 cell alveoli per unit area in chronic group experimentals as compared to the controls (Table VII). Within the acute group alone an increased alveoli count was noted for the expirementals, but this was not conclusive. Only Type 2 alveolar cells demonstrated evidence of mitosis. The number of mitotic cells did appear noteworthy in the experimental material of the chronic group, series 3.* (Index 3.4). Otherwise the evidence of increased mitosis lacked an entirely conclusive nature (Table VIII). Type 2 cells of chronic group experimental animals showed further modified characteristics. They were more compressed between the alveoli of Type 1 cells. Their alveoli were smaller generally and more irregular in size and arrangement. The cytoplasm of some cells lacked uniformity of staining and contained vacuoles. Not

*The different series are explained in Table VI.

Fig. 1.—Parotid gland, control, chronic experiment. I.H.A.B. stain ×400. Note (1) absence of mitotic division in alveolar cells; (2) normal appearance of cell nuclei, cytoplasm, and cell membranes; (3) compactness of alveoli; (4) small amount of interlobular connective tissue.

Fig. 2.—Parotid gland, sodium fluoride, chronic experiment. I.H.A.B. stain ×400. Note the evidences (A, B, and C) of cytoplasmic change and possible degeneration in alveolar cells. In comparison with the gland of a control animal (Fig. 1.) observe (1) increased mitosis of alveolar cells; (2) increase in average size of alveolar cell nuclei; (3) apparently increased separation of alveoli and amount of intralobular connective tissue. *A*, Alveolar cell nucleus of increased size. *B*, Alveolar cell cytoplasm irregularly stained. *C*, Alveolar cells lacking distinct membranes. *D*, Mitotic division of an alveolar cell. *E*, Clear unstained area in an alveolar cell.

Fig. 3.—Parotid gland, control, acute experiment. I.H.A.B. stain ×400. Observe the homogeneous appearance of the cell cytoplasm, the prominence of the cell membranes, and the absence of mitosis.

Fig. 4.—Parotid gland, sodium fluoride, acute experiment. I.H.A.B. stain ×400. Note the evidences of experimentally produced change in the alveolar cells. *A*, Cell nucleus of unusually large size. *B*, Unstained vacuole-like area in an alveolar cell. *C*, Mitotic division. *D*, Irregular staining of cytoplasm.

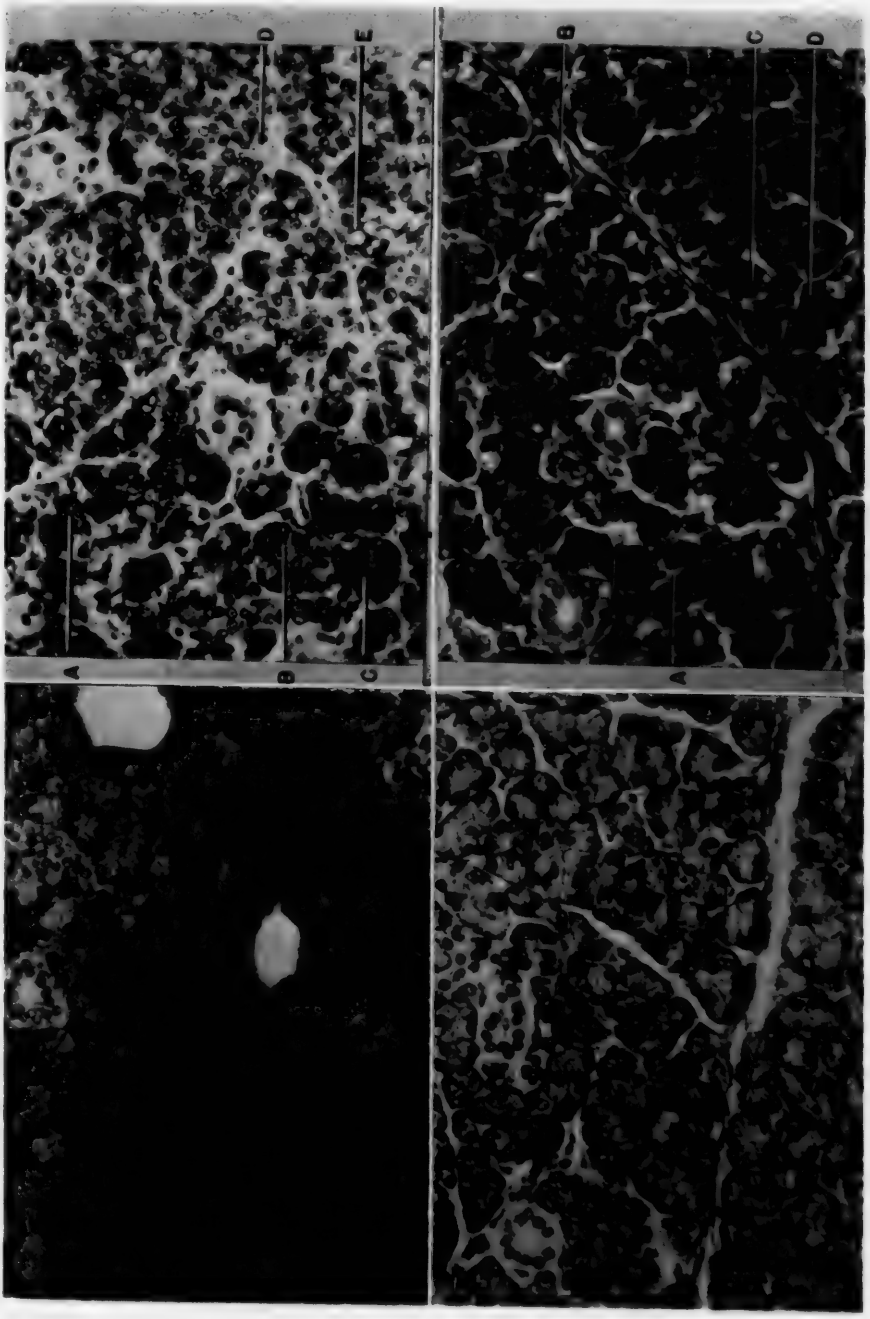

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

For legend see opposite page

only did the nuclei of separate cells present outlines which were often less well defined but also in many cells the nuclei were enlarged and of irregular shape. It was in the latter cells that the cytoplasm was of the most irregular character. Type 2 cells of the acute group exhibited to a lesser degree all the changes just reported with the exception that their close compression between the alveoli of Type 1 cells was undetectable.

Sublingual gland:—The I.H.A.B.-Methyl green stain was used as a specific means of studying the mucous cells of this gland. Mallory-Azan staining provided a further aid in the distinction of mucous substance.

No evidence of histological change was detected in the glands of the experimental animals of one group (acute or chronic) as compared with (a) the controls of the same group or (b) the control and experimental glands of the other group.

DISCUSSION

There is general agreement upon the fact that the growth curve of young animals is a sensitive index of the existence and degree of chronic fluoride intoxication.[3, 43, 44] The loss of weight attending fluoride absorption varies (depending on dosage) from a slight reduction to a rapid decline which continues until death.[3]

The lack of truly significant evidence of weight decline among surviving experimental animals (Table III) weakens the value of the present study. Despite their age uniformity, individual members of each group differed widely in their initial weight, yet the same volumes of solution were administered to each animal throughout the experiment. Exact correlation of body weight and sodium fluoride intake was therefore never achieved.

It is very difficult to produce in an entire group of animals the weight loss and cachexia of fluoride poisoning and still keep all the animals alive. The death of some experimental rats together with the steady weight increase of others proves this point. Evidently a wide range of susceptibility to fluoride exists, not only where actual survival is concerned, but also with reference to change in particular tissues. The varied degrees of mottled enamel which were

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Fig. 5.—Submaxillary gland, control, acute experiment. I.H.A.B.-Methyl green stain ×400. Note (1) comparative uniformity of cytoplasmic staining in Type 2 cells; (2) regular size of Type 2 cell nuclei; (3) proportion of Type 1 cell alveoli to alveoli made up of Type 2 cells. *A*, Alveolar cell, Type 1. *B*, Alveolar cell, Type 2.

Fig. 6.—Submaxillary gland, sodium fluoride, acute experiment. I.H.A.B.-Methyl green stain ×400. Compare with Fig. 5. Observe (1) the altered appearance of certain Type 2 cells, and (2) the increased number of Type 1 cell alveoli per unit area. *A*, Type 1 cell alveolus. *B*, Alveolus made up of Type 2 cells irregular in their size and arrangement. *C*, Type 2 cell showing vacuolation. *D*, Type 2 cell showing irregularity of cytoplasmic staining. *E*, Enlarged nucleus of a Type 2 cell.

Fig. 7.—Submaxillary gland, control, chronic experiment. I.H.A.B.-Methyl green stain ×400. The arrangement and uniformity in size of Type 2 cell alveoli, as well as the homogeneous cytoplasm and distinct cell membranes of Type 2 alveolar cells, can be readily observed. *A*, Type 2 alveolar cell. *B*, Type 1 alveolar cell.

Fig. 8.—Submaxillary gland, sodium fluoride, chronic experiment. I.H.A.B.-Methyl green stain ×400. Compare with the gland section from a younger experimental animal (Fig. 6) and observe the greater number and size of Type 1 cell alveoli in the adult gland. This difference is apparently a factor of age alone. Compare with Fig. 7 and note (1) the irregular size and arrangement of Type 2 cells and their compression between Type 1 cells; (2) the smaller average size of Type 2 cells; (3) the variations in the size of nuclei and appearance of the cytoplasm of these cells. *A*, Small nuclei of compressed Type 2 cells. *B*, Abnormally large Type 2 cell containing enlarged nucleus. *C*, Vacuolated cytoplasm of a Type 2 cell.

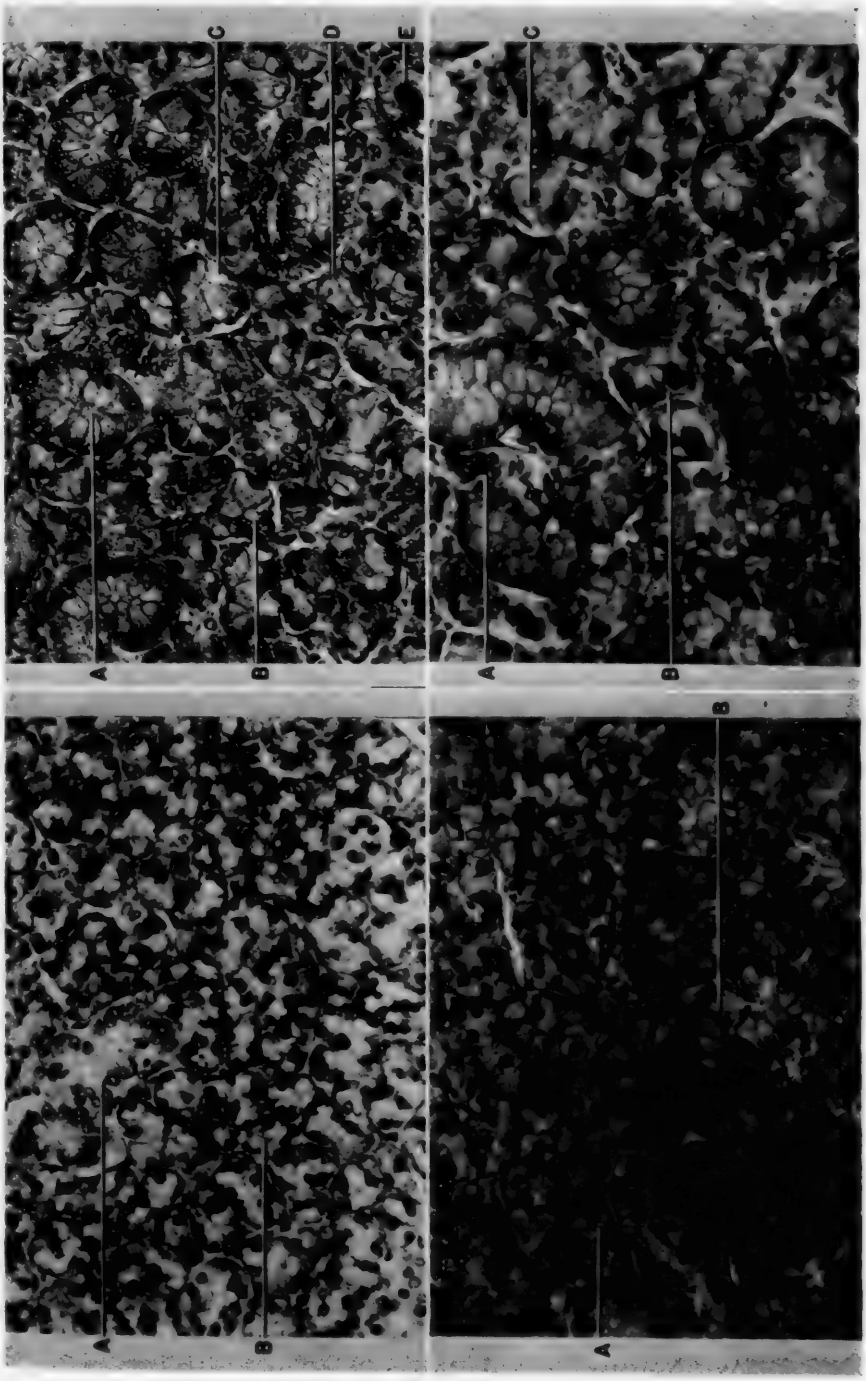

Fig. 6. Fig. 8.

Fig. 5. Fig. 7.

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observed bear this out. Future studies, therefore, should be made on the basis of a dosage level correlated to animal weight through the entire length of the experiment.

Further complicating the experimental dosage issue is the evidence of "adaptive powers of the organism to fluorine." Lawrenz, Mitchell and Ruth[45] mentioned this when they noticed a more efficient fluorine elimination in rats over lengthening periods of fluorine feeding. Intraperitoneally-absorbed fluoride must be excreted in large measure by way of the kidneys, just as the ingested salt is known to be.[5, 46] It would seem logical to assume, therefore, that an adaptation to fluorine exists, partly as a result of more efficient elimination, whenever the element is absorbed continuously at low levels.

The absence of inflammation in the peritoneal tissues of experimental animals and the prevalence of lesions at the injection site is readily explained. Often it was not possible to avoid leakage of drops of fluoride solution from the needle tip as the needle penetrated the skin and subcutaneous tissues. Furthermore, while extraneous solutions in the peritoneal cavity undergo rapid dilution with peritoneal fluid and are quickly absorbed, subcutaneous tissues lack a rapid means of fluid elimination. Once fluoride ions are accidentally introduced into these areas, they remain to exert their toxic effects at close range for some time.

The differences which exist between parotid gland tissue of acute group animals as compared to such tissue of the chronic group may be explained on the basis that the gland was undergoing development; hence the numerous mitotic cells, the small cell nuclei, and the many proliferating ducts. Such features would not characterize the mature glands.

Significant experimental increases were recorded in the number of mitotic alveolar cells of the parotid glands. This observation may point to an initial

TABLE V

PAROTID GLAND: SIZE OF ALVEOLAR CELL NUCLEI

EXPERIMENT	CONTROL				F-INJECTED				INDEX
	ANIMAL	TOTAL AREA*	$M \pm s_M$	s	ANIMAL	TOTAL AREA	$M \pm s_M$	s	
	GH00	319			BH21	370			
	B73	345			BH09	372			
Acute	W96	323	329 ± 8.09	11.43	G69	334	355.6 ± 6.45	14.43	2.57
					GH04	361			
					G96	357			
					B25	339			
	B72	345			B45	399			
	G57	322			GH16	403			
Chronic	G87	371	346.0 ± 13.57	19.165	B32	311	410.2 ± 8.195	16.41	9.74
					GH50	413			
					BH38	525			
Acute			329 ± 8.09	11.43					5.25
Chronic			346.0 ± 13.57	19.165					
Acute							355.6 ± 6.45	14.43	5.24
Chronic							410.2 ± 8.195	16.41	

*Each number in columns 3 and 7 represents the total cross sectional areas of fifty alveolar cell nuclei. The nuclei were drawn as found across the diameter of several oil immersion fields (Ocular 10×, Objective 90×, Magnification 900), these fields being in different areas of the section. Camera lucida apparatus and planimeter were employed.

TABLE VI

SUBMAXILLARY GLAND: NUMBER OF INTERLOBULAR AND INTRALOBULAR DUCTS

EXPERIMENT	SERIES	GROUP	NUMBER OF DUCTS* M ± s_M	NUMBER OF DUCTS* s	INDEX OF SIGNIFICANCE OF DIFFERENCE
Acute	1	Control	12.0 ± 1.92	3.85	2.8
Chronic	1	Control	6.5 ± 0.51	1.025	
Acute	1	F-Injected	13.86 ± 1.05	2.75	7.98
Chronic	1	F-Injected	4.2 ± 0.61	1.22	
Acute	1	Control	12.0 ± 1.92	3.85	0.85
		F-Injected	13.86 ± 1.05	2.75	
	2	Control	15.33 ± 3.85	5.44	0.62
		F-Injected	12.67 ± 1.88	1.72	
Chronic	1	Control	6.5 ± 0.51	1.025	2.90
		F-Injected	4.2 ± 0.61	1.22	
	2	Control	8.2 ± 1.45	2.92	1.05
		F-Injected	6.5 ± 0.74	1.71	
	3	Control	6.0 ± 1.53	1.25	0.147
		F-Injected	6.33 ± 1.62	2.28	

*Four high power fields (Ocular 5×, Objective 40×, Magnification 200.) were examined in each slide and a count made of inter- and intra-lobular ducts visible in all planes of view. *Series 1* represents tissue fixed in Zenker formol and stained with I.H.A.B.-Methyl Green; *series 2*, Zenker formol fixation and Mallory Azan stain; *series 3* Bouin fixation and I.H.A.B.-Methyl Green stain.

stimulation of the cells, prior to a later poisoning of them. The greater size of alveolar cell nuclei may also indicate a generalized response to the toxic substance.

Many parotid gland alveolar cells of animals which received sodium fluoride showed evidence of change, possibly of a degenerative nature. The cytoplasm of numerous cells contained unstained circular areas suggestive of fat droplets and of fatty degeneration. It is realized that cloudy swelling (albuminous degeneration) is the usual pathologic condition accompanying a generalized toxemia and resultant disturbance of cell metabolism.[47] Fluoride poisoning, moreover, brings about both these states. Yet the swelling of individual cells

TABLE VII

SUBMAXILLARY GLAND: COUNT OF TYPE 1 CELL ALVEOLI*

EXPERIMENT	SERIES	GROUP	NUMBER OF THE ALVEOLI** M ± s_M	NUMBER OF THE ALVEOLI** s	INDEX OF SIGNIFICANCE OF DIFFERENCE
Acute	1	Control	51.6 ± 10.00	20.004	5.53
Chronic	1	Control	111.2 ± 3.97	7.97	
Acute	1	Control	51.6 ± 10.00	20.004	1.87
		F-Injected	80.14 ± 11.56	24.36	
	2	Control	71.67 ± 17.235	28.32	.65
		F-Injected	83.5 ± 6.145	13.74	
Chronic	1	Control	111.2 ± 3.97	7.97	7.69
		F-Injected	178.4 ± 7.80	15.60	
	2	Control	124.0 ± 3.44	5.96	8.70
		F-Injected	188.67 ± 6.59	14.74	
	3	Control	109.0 ± 8.125	11.49	4.87
		F-Injected	174.3 ± 10.72	23.98	

*For description of the series, see Table VI.

**Six high power fields were examined in each slide. The count indicates all the alveoli entirely or partially visible in the six fields (Ocular 5×, Objective 40×, Magnification 200).

TABLE VIII. SUBMAXILLARY GLAND: CELL MITOSIS COUNT IN ALVEOLAR CELLS*

EXPERIMENT	SERIES	TYPE	GROUP	NUMBER OF MITOTIC CELLS		INDEX OF SIGNIFICANCE OF DIFFERENCE
				$M \pm s_M$	s	
	3	2	Control	0.4 ± 0.24	0.49	0.90
Acute	1	2	F-injected	1.29 ± 0.475	1.16	
	1	2	Control	1.2 ± 0.59	1.195	2.02
Chronic			F-injected	3.2 ± 0.80	1.6	
	3	2	Control	0.67 ± 0.27	0.46	3.4
			F-injected	2.60 ± 0.50	1.02	

*For a description of the series, see Table VI.

A count was taken of the total number of mitotic alveolar cells of each type visible in ten high power fields (Ocular 10×, Objective 40×, Magnification 400) of each slide. The mean (M) represents the arithmetic mean of such counts for a series of slides in each group.

and the granularity of their cytoplasm, classic signs of cloudy swelling, were absent in this parotid gland material. Roholm did note these very cellular changes of cloudy swelling when he reviewed the degeneration of parenchymatous organs during fluorosis.[3] Parotid gland observations from this present study, therefore, do not find a parallel in the work of Roholm or in other written reports.

The sharp histological picture of cell derangement in the parotid and submaxillary glands may be found to stem from the adverse effects of fluoride upon metabolic systems within the cell. Glandular tissues have a secretory function which is made possible by the exceptional metabolic activity of their many component cells. Fluoride can render normal cell activity impossible when it inhibits the enzyme enolase and disrupts glycolysis at a critical stage. It prevents optimum cellular oxidation by blocking the reformation of adenosine triphosphate. Furthermore, it is a known inhibitor of lipase and of some esterases. Conceivably the normal lipase stimulus to the transfer of fat across the cell membrane as fatty acid and glycerol is removed by fluoride.[48] If inhibition of oxidase is likewise effective,[17] a biochemical reason for fatty degeneration would be established.

Earlier studies suggest that salivation is increased in animals both acutely and chronically poisoned with fluoride.[3, 25] Because of the work of Roholm[3] we also know that the point of attack upon salivary glands is peripheral, i.e., in the gland cells proper or in the adjacent nerve ends. Certainly it was evident in this study that the actual cells were adversely influenced by fluoride. Moreover, the survey of the parotid gland revealed that the average alveolar cell of experimental tissue included more granules. Possibly these facts and the other parotid gland findings as to the size of individual alveoli and their apparently greater separation from one another, are significantly related. They might indicate that the cells of the gland, under stimulus, were actually secreting at a faster than normal rate, and were thus never able to reach usual dimensions.

Submaxillary gland observations were in no sense identical with those made upon the parotid gland. Certain of the changes may be ruled out because of their restricted occurrence. The prolonged (chronic) influence of fluoride upon the Type 2 alveolar cells was, however, such as to produce noticeable changes both in the alveolar arrangement of these cells and in their cytoplasm and

nuclei. Just as in the case of the parotid gland cells, extensive vacuolation, absence of granulation, etc., indicated a fatty degeneration rather than a cloudy swelling.

The extent of the deviation from normal was greater in this submaxillary cell than in any other cell type of the eight organs surveyed.* Why this was so, and why the immediately adjacent Type 1 cells were not similarly affected, is not known. Type 1 cells did reflect what can be assumed was the stimulus of plasma fluoride, but in an entirely different way. The marked increase of Type 1 serous cells might possibly produce a faster salivary secretion in the experimental rats of the chronic group and a significant change in the normal ratio of salivary constituents. The observations suggest that this may be the case.

If the influence of fluoride upon the cells of one salivary gland is as described, there still remains to be answered the question of the sublingual gland and the entire absence of obvious cytological change in its structure. Handler's description of intense, thick salivation in lethally poisoned rats[15] would suggest that under certain conditions the mucous salivary alveoli are either highly stimulated or altered in function. The mucous cells of the sublingual may be fluoride-resistant because of the particular nature of their secretion or of its synthesis. Further studies of the sublingual gland tissues under the influence of NaF are indicated in order to substantiate this hypothesis.

Questions of carbohydrate digestion in the mouth and the metabolism of oral bacteria are both linked to these histological findings. Apparently serous cells of both the parotid and submaxillary glands are specifically injured by the fluoride ion. There is reason to believe that the enzymic content of the saliva is changed as a result of disrupted secretion in these cells. Study of salivary amylase has shown that the enzyme is not inhibited by fluoride acting upon it in vitro.[13] Yet this is not conclusive evidence in support of a negative effect upon secretion. Neither does it indicate absence of change in the over-all salivary enzyme content during fluoride absorption.

SUMMARY

1. Two groups of rats were studied, each group consisting of ten experimental and five control animals. The two groups were designated as "acute" and "chronic," based on the duration of the experimental period and amount of sodium fluoride administered. The acute group animals received daily intraperitoneal injections of 2.5 per cent isotonic aqueous NaF solution for 15 days, while the chronic group experimental animals received lower volume injections of the same solution on alternate days over a 100-day period.

2. At autopsy, no significant changes were observed either in the gross appearance of the animals (incisor teeth excepted), or in the gross structure of the salivary glands.

3. The chief histological effects of sodium fluoride administration, confirmed in many instances by statistical analysis, were as follows:

*The findings in the kidney, liver, pancreas, adrenal gland and thyroid gland are being reported separately.

Parotid Gland.—(1) Increased mitotic division of alveolar cells (chronic group experimental animals), (2) increased size of alveolar cell nuclei (chronic group experimental animals), (3) clear unstained areas and a lack of uniform staining in the alveolar cell cytoplasm, features suggestive of degenerative change. There were reasons for considering this a fatty degeneration, and (4) increased separation of alveoli and an apparent increase in the amount of intralobular connective tissue.

Submaxillary Gland.—(1) An increase in the number of Type 1 serous cell alveoli per unit area (chronic group experimentals). The Type 1 cell is described by Stormont[36] as a cell containing large highly refractive granules, (2) a modification of serous cells of the second type (Type 2), including a change in their size and alveolar arrangement and their greater compression between alveoli of Type 1 cells, and (3) evidence of cytological change in Type 2 cells. Vacuolation and unusual staining of the cytoplasm and the enlarged, irregular shape of many cell nuclei pointed to degeneration, possibly of a fatty nature. These Type 2 cell changes were apparent, though to a lesser degree, in submaxillary glands of the acute experimental group.

Sublingual Gland.—Experiment tissues were devoid of change.

The effects of sodium fluoride absorption were such as to produce definite change in the structure of the parotid and submaxillary glands. Similar dosage of sodium fluoride did not influence the histological appearance of the sublingual gland.

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RADIOIODINE PENETRATION THROUGH INTACT ENAMEL WITH UPTAKE BY BLOODSTREAM AND THYROID GLAND

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INVESTIGATIONS conducted to determine whether substances will penetrate enamel centripetally have resulted in conflicting reports (Fish[1] and Berggren[2]) as to whether large dye molecules and silver nitrate and other reagents can penetrate enamel from its surface (centripetal penetration). Centrifugal penetration of dyes into enamel (from the pulpal side) has also been described. Definitive evidence of rapid dye penetration through enamel in either direction is not available. Centripetal experiments (Atkinson[3]) indicate salts penetrate the enamel of extracted teeth. Wainright and Lemoine[4] showed centripetal penetration of radiocarbon-labelled urea through intact enamel of extracted teeth. The possibility of differences in permeability of extracted teeth as opposed to teeth in situ has been reported by Bodecker and Lefkowitz.[6]

Berggren,[2] using in vivo methods, demonstrated centripetal penetration of P-32 and tetanus toxin through enamel. Mandel and Sarkady[5] administered large doses of sodium iodide to cats and showed some evidence of penetration of the iodide into the enamel and dentin. Bartelstone and co-workers,[7, 8] demonstrated radioautographic evidence of penetration of I-131 into enamel in cats and human beings following systemic administration of the isotope.

This study was undertaken to determine whether I-131 applied to the intact surface of enamel would penetrate through the enamel and dentin and concentrate in the thyroid gland of cats.

METHOD

Eight adult male cats, each weighing 6 to 8 pounds, were used. The animals were maintained under Nembutal anesthesia throughout the experiment.

A wax block was prepared by folding together warmed sheets of dental base plate wax (Fig. 1). The size of the wax block prevented movement of the mandible. Two aluminum shells were inserted into the dorsal surface of the wax block, the distance between the center of the shells equalling the interval between the maxillary canines.

A solution (0.5 ml.) containing about 500 microcuries of carrier-free I-131, in the form of NaI, was placed in each shell. The teeth were wiped with surgical sponges and the wax block placed between the jaws (Fig. 1) in such

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a manner that only the tips of the teeth were dipped and retained in the solution. When the maxillary canines were used as experimental teeth, the mandibular canines acted as controls. The cat was placed on a lead plate, 1 inch thick, and its head surrounded by lead blocks. A portable shielded Geiger counter was fixed at the level of the thyroid cartilage. To reduce the background count because of the I-131 source in the mouth, lead sheets were positioned immediately inferior to the mandible. The counting rate over the thyroid gland was determined to establish the background as a zero point at the start of the experiment. The experiment was continued until significant counts, above the background, were noted.

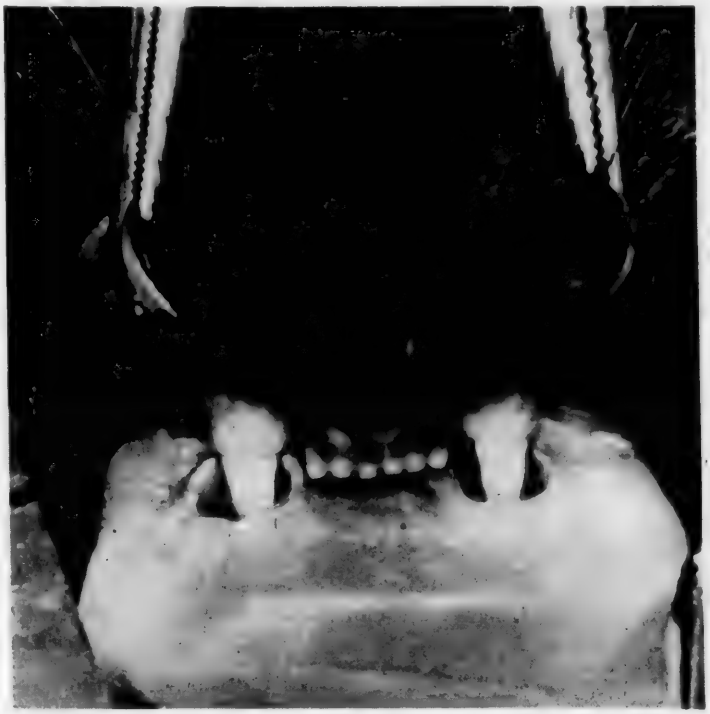

Fig. 1.—Tip of canines of anesthetized cat dipping into crucibles containing 0.5 ml. NaI-131 (500 microcuries). Note that rubber dam although normally in position isolating canines, has, in this case, been removed for photographic purposes.

The animals were sacrificed and longitudinal slices, in a sagittal plane, were cut from the four canines and the supporting tissue of each. Tooth sections were ground to seventy-five micra under oil (oil reduces leaching of I-131),[7] and buffed and polished until free of contaminated grinding debris. Frozen sections of the thyroid glands were prepared.

Radioautographs were made from the tooth and thyroid sections on Kodak contrast lantern slide plates and No-Screen x-ray film. Exposure was estimated by Geiger counter surveys, assuming uniform distribution of I-131 as well as uniform self-absorption of sections.

RESULTS

Activity over the thyroid gland after 1.5 to 2 hours as demonstrated by Geiger counter and radioautographs was evidenced in all cases.

Centripetal penetration of I-131 into the tooth is shown in the radioautograph (Fig. 2). The distribution pattern indicates a diffuse penetration of I-131 through enamel with a concentration of radioiodine at the dentino-enamel junction. Dentin is diffusely penetrated and I-131 is found in the pulp (Figs. 2*B*, and 3*B*).

There is radioautographic evidence (Fig. 2, *B*) of distribution of I-131 in cementum, periodontal membrane, alveolar bone and marginal gingiva. In the radioautograph illustrated (Fig. 2, *B*) there is no evidence of I-131 distributed on that portion of the enamel surface which was not actually dipped into the source solution.

No evidence of I-131 uptake was found in any of the control sections.

DISCUSSION

Radioiodine was chosen as the tracer substance because it has been found useful for studying the distribution of tissue fluid solutes in teeth of human beings and cats.[7, 8] A relatively high concentration of I-131 is found in saliva following systemic administration.[8]

The method devised for this experiment has several advantages over previous methods for applying reagents to teeth. It may be used for both centripetal or centrifugal studies of enamel permeability. The external environment of the expermental tooth can be controlled, permitting variation in the type of fluid, temperature, and level of immersion. No time limit is imposed by this method. The introduction of variables, such as cavity preparations and cements or waxes, are not required to keep the reagent in contact with the tooth.

There was no evidence of surface spread of I-131 beyond the level of the solution. The bare area corresponding to the enamel surface seen in Fig. 2, *B* demonstrates this point. It may therefore be deduced that distribution in the periodontium is not due to any external bridge between the I-131 source and the mucous membrane.

Survey radioautographs were used. Although automatically superimposed autographs and sections would be preferable, the tooth sections were too thick to permit collimation of radiation to a degree commensurate with microscopic observation at high power. Because these ground sections were unmounted, a variation from the conventional survey radioautographic technique was employed. The sections were placed between two emulsions varying in sensitivity to radiation. One exposure produced two radioautographs clearly visualizing areas of low uptake on the fast emulsion and areas of high uptake on the slower emulsion.

By using the thyroid gland as an indicator, the systemic uptake of I-131 was demonstrated earlier than if the blood had been counted. If the experimental time interval had been extended to obtain detectable concentration of I-131 in the blood, the control teeth would have given positive radioautographs.

This would have made it impossible to study the I-131 distribution patterns through the tooth and periodontium as a function of the source external to the enamel surface.

The radioautographs of the tooth sections may be considered analagous to the dynamic interpretation of candid photography of moving objects. The distribution patterns demonstrate the penetration through the tooth of I-131 "stopped" at a given point in time as it proceeds toward the pulpal tissue from the surface. No detectable changes in the distribution of the I-131 were found in successive radioautographs made from the sections indicating that I-131 did not shift in the tooth after sectioning.

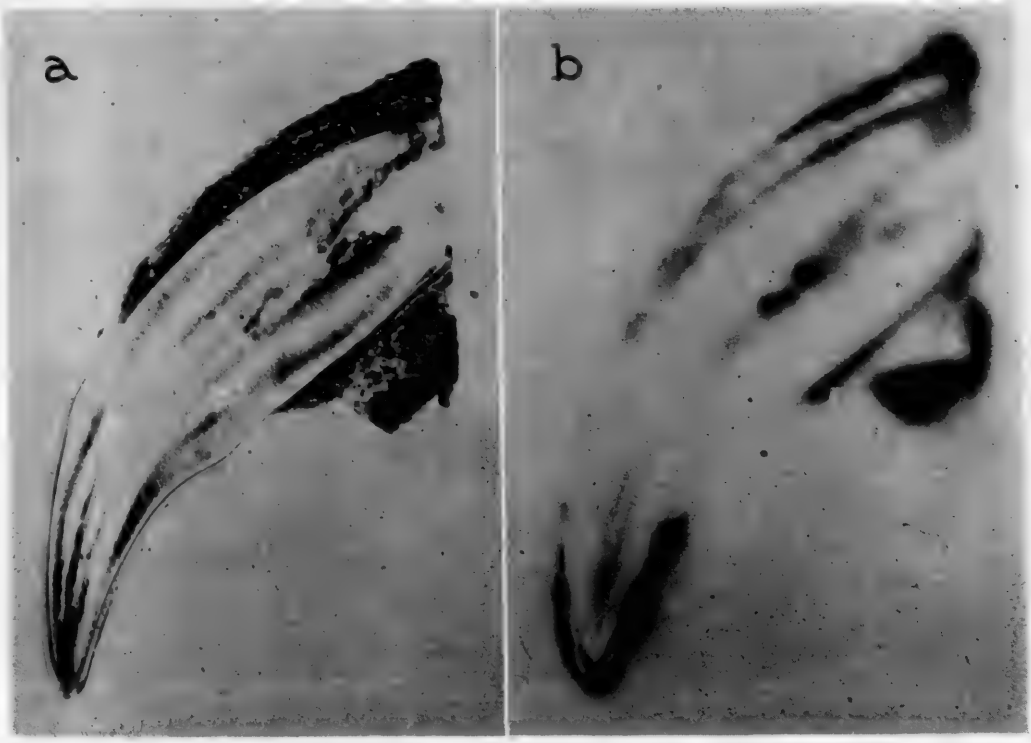

Fig. 2.—*A*, Photomicrograph of 75 micra longitudinal ground section of cat lower canine (experimental). *B*, Radioautograph made from section in *A* on Kodak contrast plate. Distribution of I-131 through enamel and into dentin. Uptake by cementum, periodontal membrane, alveolar bone, and gingiva. Absence of I-131 contamination on enamel surface, apically, beyond level of solution.

In I-131 studies previously reported,[7, 8] in which the iodide was administered systemically, uptake was obtained in the cementum, bone, and connective tissue of the periodontium. Under conditions imposed by the present experiment, however, the distribution pattern in the periodontium is considered significant because the evidence indicates a pathway, other than systemic approach, for penetration of a tissue fluid solute into the periodontium by way of the tooth. The degree to which this pathway of entrance may influence the periodontium remains to be determined.

Fig. 4 suggests that some of the I-131 enters the periodontal membrane after leaving the pulp, and reaches the alveolar bone and cementum. However, upon leaving the pulp, the I-131 may enter the bone at the apex of the root of the tooth, and after distribution in the bone, reach the periodontal membrane, cementum, and gingiva. The lateral spread of I-131 along the dentino-enamel junction does not appear to be a pathway to the periodontium under the conditions of the experiment.

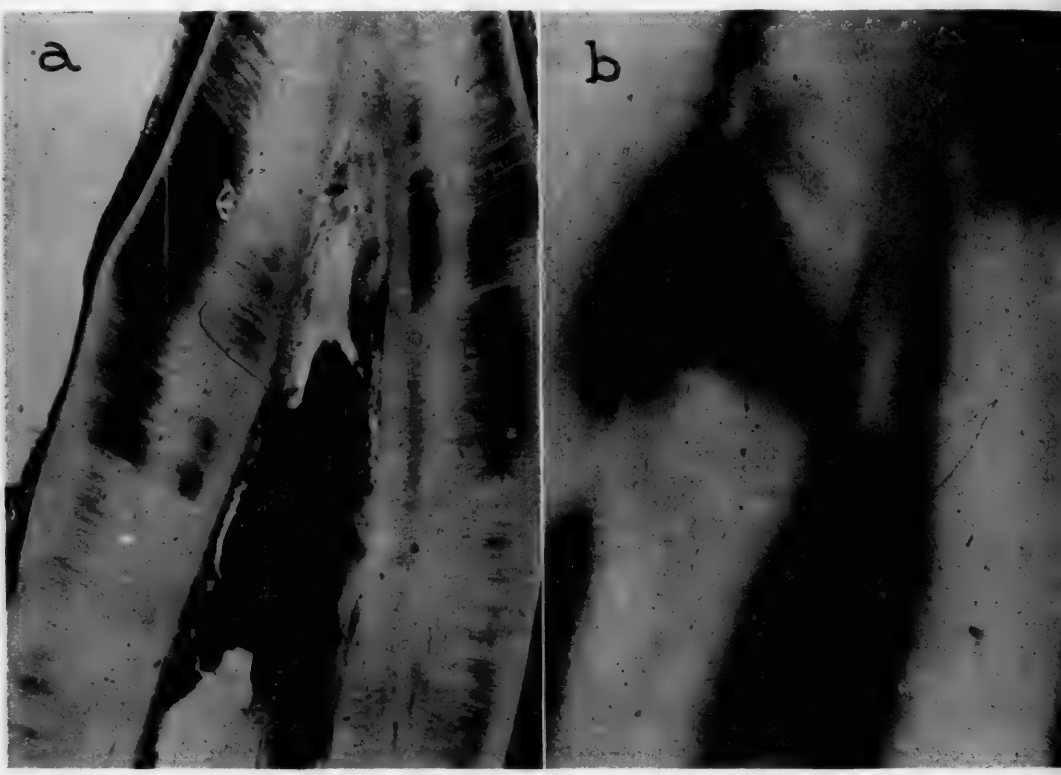

Fig. 3.—*A*, Photomicrograph of cervical one-third area of 75 micra longitudinal ground section of cat upper canine (experimental). Note darkened tubules in dentin, presence of some pulp tissue. *B*, Radio-autograph made from section in *A* on Kodak contrast plate. Diffuse penetration through enamel and dentin, extending into pulp.

SUMMARY

A solution (0.5 ml.), containing 500 microcuries of carrier-free I-131 as NaI-131, was applied to the external surface of intact enamel of eight animals. Significant counts were observed over the thyroid gland, indicating penetration of I-131 through enamel and dentin and thus into the pulp and the blood-stream. Radioautographs demonstrate diffuse penetration of I-131 through enamel and dentin and uptake by the periodontal tissues.

Grateful acknowledgment is extended to Drs. Bernard Roswit and Rosalyn Yalow of the Veterans Administration and Dr. Barnet Levy of Columbia University for their cooperation

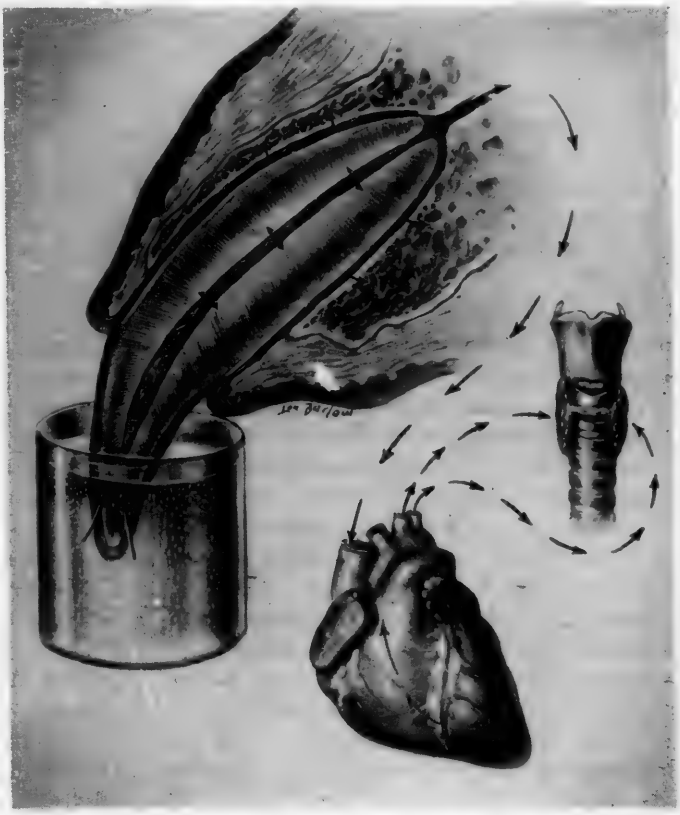

Fig. 4.—Penetration on I-131 from source in crucible, through intact enamel and dentin into the pulp and periodontium. Uptake of I-131 by bloodstream with concentration in thyroid gland.

and advice; to Mr. Martin Jachter and Mrs. Katherine Newerby for technical assistance, and to Mr. Sidney Shapiro and Mr. Lou Barlow of the Medical Illustration Department of the Veterans Administration.

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CORRELATION OF HYPERCEMENTOSIS WITH TOXIC GOITER

A Preliminary Report

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THE etiology and significance of hypercementosis is at present still under investigation. Zemsky[1] pointed out the hereditary factor as exemplified in a case of a mother and her two adult daughters all showing radiological evidence of hypercementosis on the roots of otherwise normal teeth. Gottlieb[2] found spikelike cementum hyperplasias on teeth under increased stress caused by a heavy pipe in an habitual pipe smoker. Boyle[3] pointed out that cementum hyperplasia represented "a defense reaction of the organism" to a low grade irritation in pulpless teeth. Kellner,[4] however, presented evidence that increased function does not cause hypercementosis. Gardner and Goldstein[5] after surveying material at the Mayo Clinic, concluded that hypercementosis represented "an inherent tendency of certain persons rather than . . . a hyperplastic response to infection only . . ." More recently, Sorrin[6] concluded that there is no relationship between hypercementosis and arthritis. Stafne[7] noted that toxic goiter in the adult may be correlated with osteoporosis, but concluded that the degree of osteoporosis does not vary significantly to be of diagnostic value.

The purpose of this paper is to present data indicating a correlation between hypercementosis and toxic goiter.

Hypercementosis is defined by Orban[8] as "an abnormal thickening of the cementum. It may be diffuse or circumscribed, i.e., it may affect all the teeth or only certain parts of one tooth." Thoma and Goldman[9] noted that it may occur in teeth that are in normal occlusion, and with no occluding antagonist as well as nonerupted teeth.

The cases of toxic goiter have been obtained from the Women's Thyroid Ward of the Kings County Hospital. Of the 36 cases, 12 have pathological diagnoses (9 cases of diffuse and 3 cases of nodular goiter). The remaining 25 were clinically diagnosed by the Medical Service of this hospital as toxic goiter. The broad definition of Means[10] is used to include "any condition of spontaneous hypersecretion on the part of the thyroid." "Toxic" pertains to the symptoms while that of "goiter" signifies the often accompanying enlargement of the thyroid gland.

Two groups were selected as controls:

Hospital Control Group.—Thirty-six white women of identical age distribution as the toxic goiter group were chosen at random from the Fracture Clinic

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and the Physiotherapy Clinic of the Kings County Hospital. Full mouth x-ray series were then obtained.

Population-at-large Control Group.—Thirty-six full mouth x-ray series of white women were selected at random from the author's own private files. This group has the same age distribution as does the toxic goiter group. The x-rays were not read until the entire group had been selected.

The radiographic interpretation as to the presence or absence of hypercementosis was made, according to the definition above, by the author and a second impartial dentist.

Table I is a classification of the individuals in each group according to the number of hypercementosed teeth. Table II is a classification of the individuals in each group by age and prevalence of hypercementosis. The results indicate that 80 per cent of the patients in the toxic goiter group have evidence of hypercementosis as compared to 19 per cent in the hospital control group and 19 per cent in the population-at-large group.

At present, the problem is being further studied in an effort to establish the nature of this relationship between hypercementosis and toxic goiter.

TABLE I

CLASSIFICATION OF INDIVIDUALS IN EACH GROUP BY THE NUMBER OF HYPERCEMENTOSED TEETH

PATIENTS HAVING THE FOLLOWING NUMBER OF HYPERCEMENTOSED TEETH	TOXIC GOITER CASES	HOSPITAL CONTROL CASES	POPULATION-AT-LARGE CASES
0	2	30	29
1	5	1	3
2	11	3	2
3	10	1	0
4	1	1	1
5 or more	2	0	1
Known positive but unknown as to number	5	0	0
Total	36	36	36

TABLE II

CLASSFICATION OF INDIVIDUALS IN EACH GROUP BY AGE AND PREVALENCE OF HYPERCEMENTOSIS*

	TOXIC GOITER CASES		HOSPITAL CONTROL CASES		POPULATION-AT-LARGE CASES	
AGE GROUP	NUMBER OF CASES	POSITIVE FOR HYPERCEMENTOSIS	NUMBER OF CASES	POSITIVE FOR HYPERCEMENTOSIS	NUMBER OF CASES	POSITIVE FOR HYPERCEMENTOSIS
11-20	1	1	1	0	1	0
21-30	12	9	12	2	12	2
31-40	9	8	9	1	9	2
41-50	8	5	8	3	8	2
51-60	4	4	4	1	4	0
61-70	2	2	2	0	2	1
Total	36	29	36	7	36	7
Percent of hypercementosis	$\frac{29}{36} = 80\%$		$\frac{7}{36} = 19\%$		$\frac{7}{36} = 19\%$	

*These data have been analyzed by the Chi—square method and have been found significant.

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THE INFLUENCE OF ROTATIONAL SPEED ON TEMPERATURE RISE DURING CAVITY PREPARATION

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RECENTLY there has been considerable interest in the possible use of high speeds and various hand pressures as they influence the temperature rise during cavity preparation. One study has been reported in which speeds up to 60,000 r.p.m. have been used.[1] A previous report from this laboratory has described thermal changes developed during the cutting of tooth tissue where various instruments are used at speeds produced by the conventional commercial dental engine.[2] From each of these studies it is apparent that in the use of rotating instruments to cut tooth tissue, the problem of heat generation is only one part of a larger problem which also includes the efficiency, vibration characteristics, and life expectancy of the rotating cutting instrument. Inasmuch as each of these four factors is influenced by several variables such as size, design, speed of operation and pressure applied, it is apparent that the application of the rotating dental instrument to the cutting of tooth tissue is basically a complex operation.

The purpose of this study was to determine the effect of speeds up to 11,300 r.p.m. on the temperature developed in the tooth during the cutting of enamel and dentin simultaneously. For this study only one design of bur (fissure type) was employed. To a limited degree the force applied during the cutting operation and the size of the instrument was varied. These variations, however, were well within the limits of conventional practice.

METHOD OF TEST

Extracted noncarious molar teeth were used in all tests described. These teeth were stored in tap water until they were needed for tests. Each tooth was prepared to accommodate a thermocouple by cutting a hole to the dentin-enamel junction at a point approximately 1 to 2.5 mm. below the occlusal surface, using a number one round bur.

A thermocouple of 28 gauge chromel-alumel wires, connected to a Type "G" Leeds and Northrup Speedomax potentiometer-recorder, was wedged into the tooth at the dentino-enamel junction. This connection allowed temperature recordings to be made at four-second intervals.

A beam balance was constructed so that it was supported in the center and could be loaded at either end. One end was designed to hold a platform on which the teeth were mounted in plaster. The other end was counterbalanced by filling a cup with shot, in sufficient quantity to counteract the weight of the

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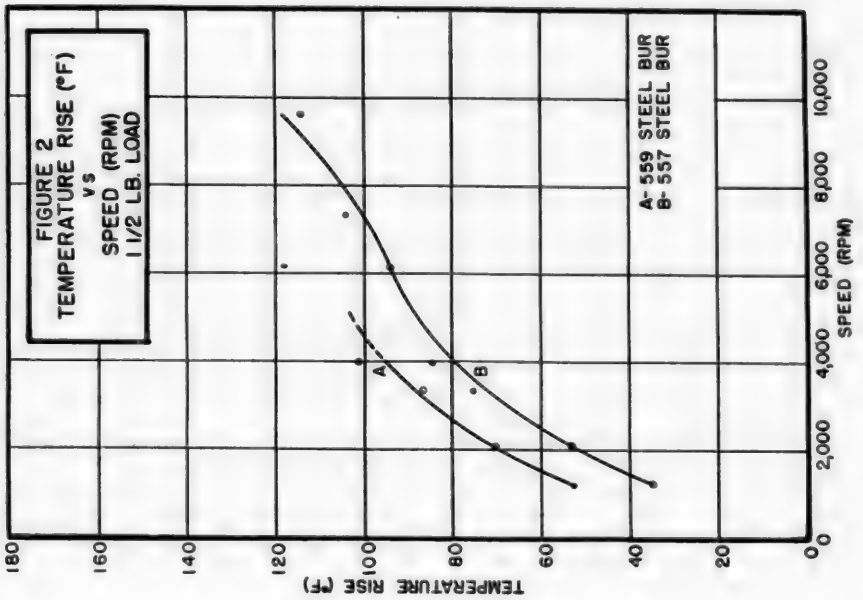


FIGURE 2
TEMPERATURE RISE (°F)
vs
SPEED (RPM)
1 1/2 LB. LOAD
A- 559 STEEL BUR
B- 557 STEEL BUR
TEMPERATURE RISE (°F)
SPEED (RPM)
0
2,000
4,000
6,000
8,000
10,000
20
40
60
80
100
120
140
160
180
A
B

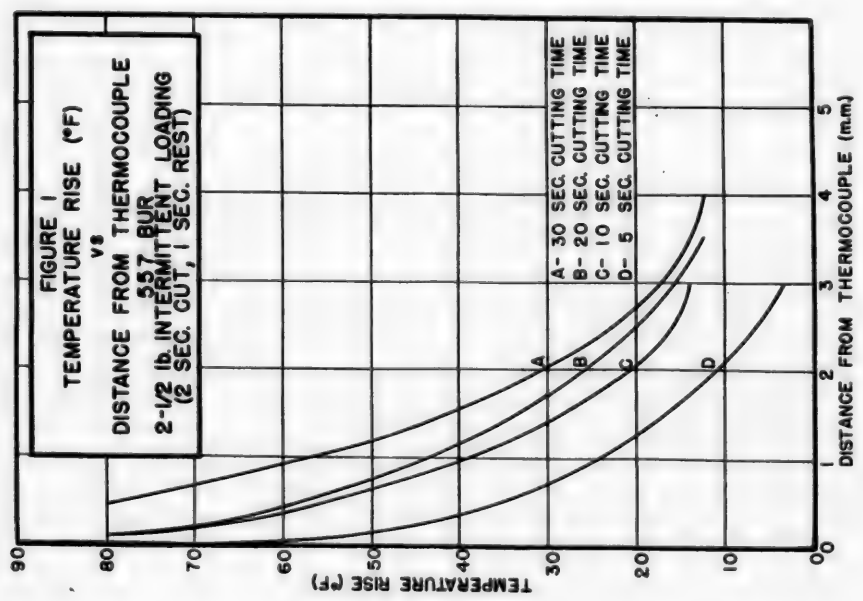


FIGURE 1
TEMPERATURE RISE (°F)
vs
DISTANCE FROM THERMOCOUPLE
2-1/2 lb. INTERMITTENT LOADING
357 BUR
(2 SEC. CUT, 1 SEC. REST)
A- 30 SEC. CUTTING TIME
B- 20 SEC. CUTTING TIME
C- 10 SEC. CUTTING TIME
D- 5 SEC. CUTTING TIME
TEMPERATURE RISE (°F)
DISTANCE FROM THERMOCOUPLE (m.m.)
0
1
2
3
4
5
10
20
30
40
50
60
70
80
90
A
B
C
D

platform, plaster, and tooth. Below this counterbalance were suspended known weights. A support was positioned so that the beam would be level before a load was applied to the tooth during the cutting operation.

A four-speed laboratory motor capable of rotating at speeds of 1,310, 2,200, 3,400, and 4,000 r.p.m. at the driveshaft was used to operate a conventional dental handpiece. By varying the diameter of the drive pulley, and using the four motor control settings, various speeds between 1,155 and 11,300 r.p.m. were obtainable at the handpiece. To cover the range of rotational speeds available, it was necessary to employ only six or eight operating speeds, such as 1,155, 2,200, 3,400, 4,000, 6,200, 7,300, 9,600, and 11,300 r.p.m.

Except for data involving tungsten carbide burs and spiral fluted burs, new burs of one product, purchased through usual trade sources, were used throughout the tests. A new steel bur was used for each measurement, so that the effect of pronounced dulling would not produce variable results. In the case of tungsten carbide burs, they were used until a microscopic comparison of used and new instruments showed the used ones to be slightly dulled. The spiral flutted steel burs described in this report were of a different make, but no comparison of various trade products is attempted. The fissure bur proved adaptable to laboratory tests for cutting enamel and dentin, and its wide application in dental practice justifies this study in detail. Throughout this report all values listed are averages of at least two, but usually several more, measurements.

DISCUSSION OF RESULTS

If the temperature rise at the surface being cut is known under a given set of conditions of operation, and if the distance of this surface from the pulp chamber can be estimated with some accuracy, an approximation of the temperature rise at the pulp can be made. For use in such determinations, the temperatures developed at various distances from the thermocouple are shown in Fig. 1, with curves for cutting times of 5, 10, 20, and 30 seconds. To obtain the 5, 10, and 20 second temperature vs. distance curves, vertical cuts in tooth structure were made from the occlusal surface of the tooth at various distances from the thermocouple. A load of 2½ pounds was used on a No. 557 steel bur for these tests. The operating speed was 3,400 r.p.m. Data for the 30 second cutting interval were obtained as described previously.[2] From these curves, it is apparent that near the heat source a large temperature rise is obtained soon after the operation begins.

Throughout the study, intermittent cutting was used. The time cycle for this was two seconds of cutting followed by one second rest. While this procedure may allow slightly greater variation in the results than if the cutting is constant, it was thought advisable to use a method which closely resembled that usually recommended in practice. The cutting time was not rigidly controlled, except for results described in Fig. 1, but the thermocouple was placed at a position such that approximately two minutes elapsed before the thermocouple was reached. The temperatures recorded were those obtained when cutting within 0.5 mm. of the thermocouple. All temperature rises were measured from

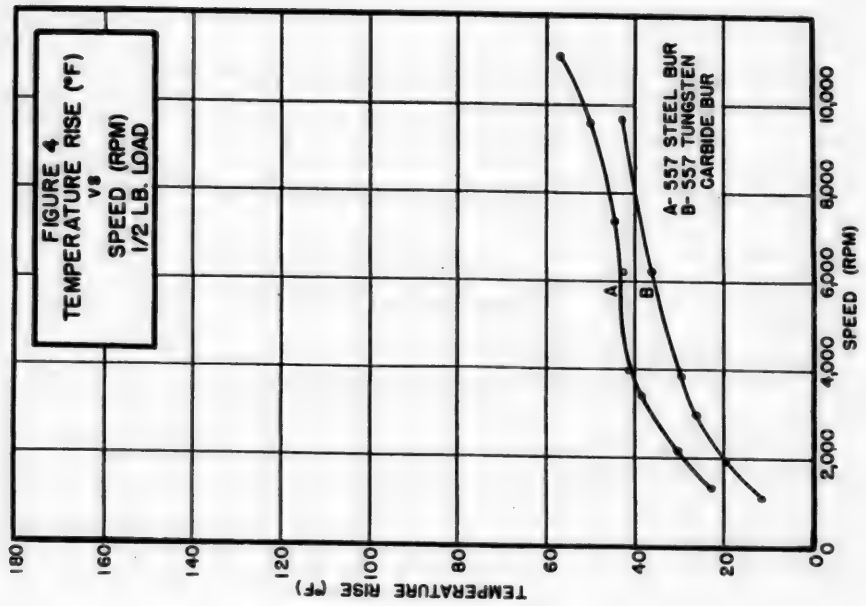


FIGURE 4
TEMPERATURE RISE (°F)
VS
SPEED (RPM)
1/2 LB. LOAD
A- 557 STEEL BUR
B- 557 TUNGSTEN CARBIDE BUR
TEMPERATURE RISE (°F)
SPEED (RPM)
0
20
40
60
80
100
120
140
160
180
2,000
4,000
6,000
8,000
10,000

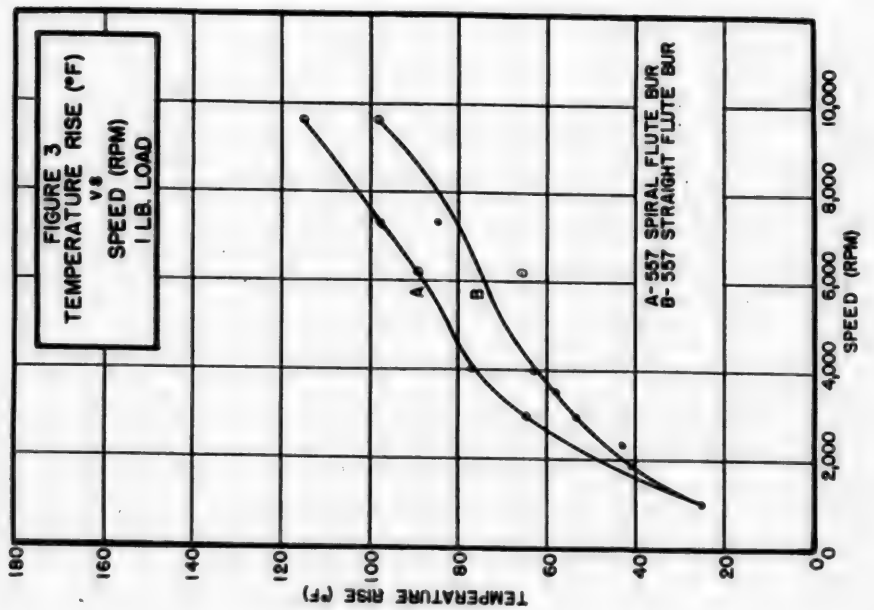


FIGURE 3
TEMPERATURE RISE (°F)
VS
SPEED (RPM)
1 LB. LOAD
A- 557 SPIRAL FLUTE BUR
B- 557 STRAIGHT FLUTE BUR
TEMPERATURE RISE (°F)
SPEED (RPM)
0
20
40
60
80
100
120
140
160
180
2,000
4,000
6,000
8,000
10,000

room temperature. From other studies to be reported later, it has been determined that the total temperature rise on extracted teeth measured as described here, is not significantly different from that obtained from teeth in the mouth.[3]

The effect of a change in bur size on temperature rise is demonstrated in Fig. 2, when using a 1½ pound load. The complete curve for the No. 559 bur was not obtained, but the trend of the curves was established by the use of the No. 557 bur at speeds between approximately 1,000 and 10,000 r.p.m. It is observed that the temperature rise of the smaller No. 557 bur is lower throughout the range tested than that of the No. 559 bur. A pronounced increase in temperature is observed as the speed is increased over the entire range tested.

The difference in the effect of straight and spiral blade or flute design as it influences temperature rise has been studied for the No. 557 bur. The temperature rises developed by the spiral flutted bur and the straight fluted bur are shown in Fig. 3, with a 1 pound load applied. The temperature rise recorded for the spiral bur was higher than that recorded for the straight flutted bur for each speed studied. It has been observed that the spiral grooved bur has greater cutting efficiency than those with straight grooves[4]; therefore under the same conditions of operation, more material will be removed per unit time with the spiral grooved bur than with a comparable straight grooved bur. In this case, the bur which operated most efficiently also developed a higher temperature rise in the tooth.

The temperature rise developed by the No. 557 tungsten carbide bur as compared to a similar steel bur is shown in Figs. 4 and 5 when operating at ½ and 1 pound loads, respectively, throughout the speed range from 1,000 to 10,000 r.p.m. The tungsten carbide bur consistently showed a slightly lower temperature rise than the steel bur when operated under comparable conditions. In another study it has been shown that the cutting efficiency of the No. 557 steel bur is similar to that of the No. 557 tungsten carbide bur.[4] It is possible that the carbide bur develops a little lower temperature rise than the steel bur because of its tendency to resist dulling. Limited data have been accumulated which would indicate this possibility.

The temperature rise developed for four constant speeds is plotted against applied load in Fig. 6. At the speeds examined it may be observed that an increase in load is accompanied by approximately a linear increase in temperature rise. At slow speeds of operation the temperature rise is approximately doubled as the load is increased from ½ to 2 pounds. At higher speeds the rate of temperature rise is even greater as the load is increased. This would support the belief of some operators that relatively light pressures and/or slow speeds are most desirable.

In Fig. 7 the temperature rise is plotted against instrument speed for four loads. The No. 557 steel bur was used for each of these tests. It is interesting to note that as the speed is initially increased the temperature rise is rapid. As the speed is further increased the temperature rise increements become smaller for a short range and then increase as the speed increases to the upper limit of 11,000 r.p.m. It appears from these results that when operating with light pressures, the temperature rise is not excessive, as compared to heavier pressures, even when high speeds are employed.

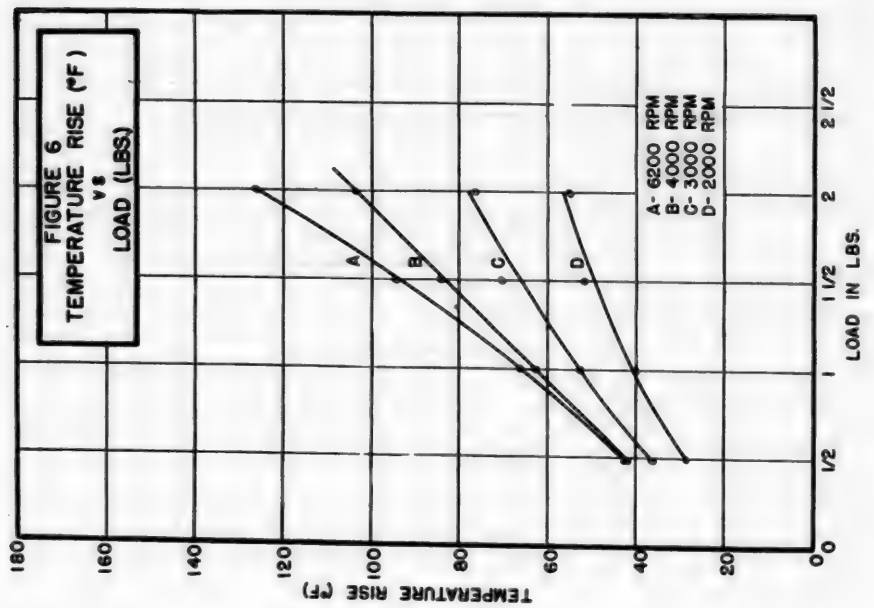


FIGURE 6
TEMPERATURE RISE (°F)
VS
LOAD (LBS.)
TEMPERATURE RISE (°F)
LOAD IN LBS.
A- 6200 RPM
B- 4000 RPM
C- 3000 RPM
D- 2000 RPM
A
B
C
D

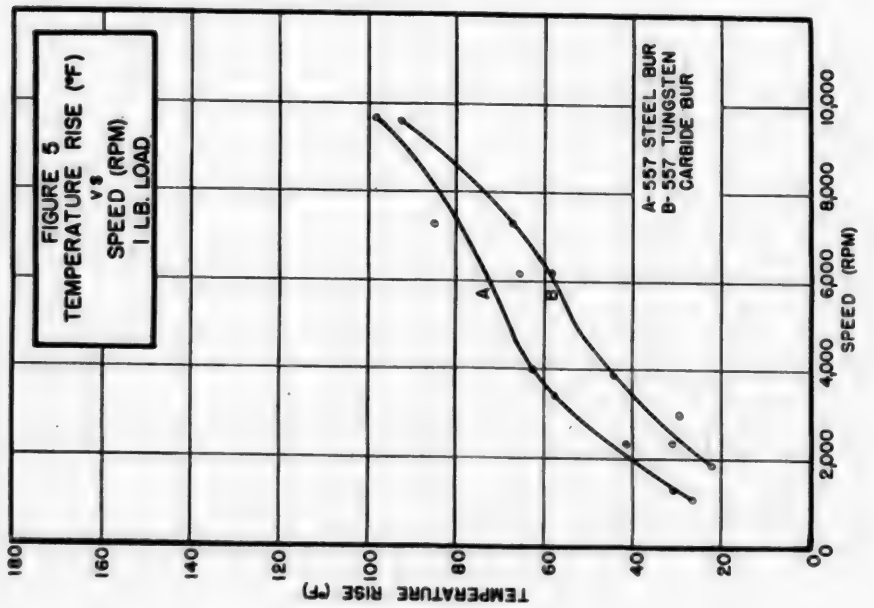
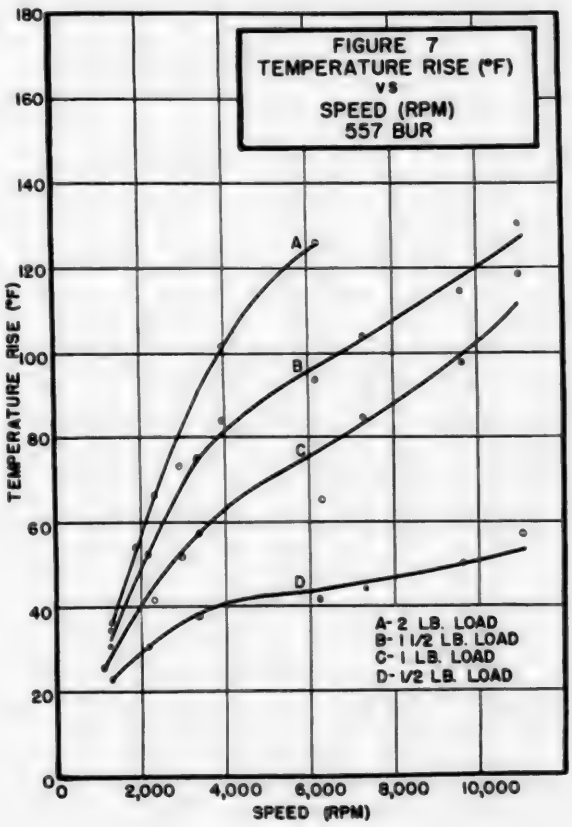


FIGURE 5
TEMPERATURE RISE (°F)
VS
SPEED (RPM)
1 LB. LOAD
TEMPERATURE RISE (°F)
SPEED (RPM)
A- 557 STEEL BUR
B- 557 TUNGSTEN CARBIDE BUR
A
B

In Fig. 8 is presented a composite three dimensional graph formed by plotting the data contained in Figs. 6 and 7. In this graph, the effect of the two variables, speed and load, upon the temperature rise may be seen.* The maximum temperature rise developed by the two pound load and a speed of approximately 11,000 r.p.m. was not completely established. Temperature rises of more than 160° F. were recorded, however, at these operating conditions, but further studies are needed to determine with certainty the peak temperatures obtainable.



CONCLUSIONS

1. Results have been presented which indicate the temperature rise within the tooth which may be expected when the fissure type bur is used at speeds between approximately 1,100 and 11,000 r.p.m.

2. From the results obtained it appears that near the heat source, or cutting instrument, the maximum temperature rise is developed within ten seconds after the operation begins.

3. The size of the cutting instrument has an influence on the temperature rise observed, and the smaller the bur used the lower the temperature rise.

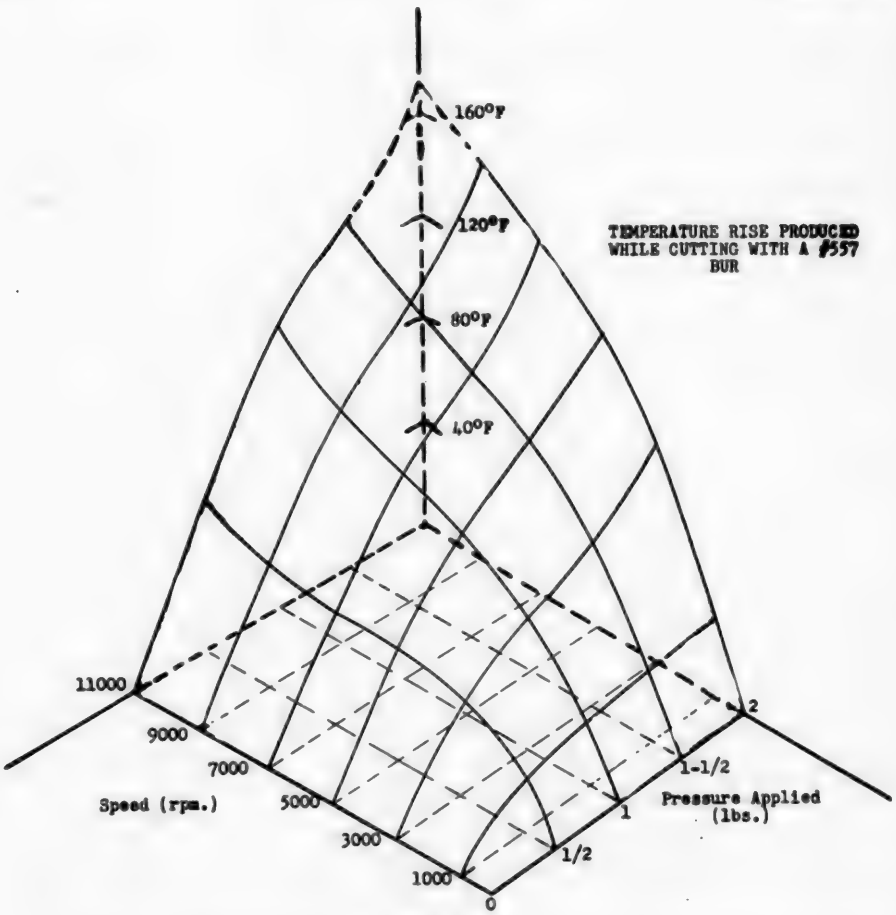


Fig. 8.*

4. The spiral fluted burs studied developed a greater temperature rise than the straight fluted burs.

5. Tungsten carbide burs were shown to produce a lower temperature rise than comparable steel burs operated under the same conditions.

6. Both the increase in pressure and increase in speed of operation were observed to have a pronounced influence on increasing the temperature rise developed in the tooth by all cutting instruments tested.

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*The authors acknowledge the assistance of Mr. E. E. Henry for the preparation of Fig. 8.

THE EFFECT OF SECTION SIZE ON THE MECHANICAL PROPERTIES OF WROUGHT GOLD WIRES

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RESULTS of tensile tests made with the nonprecious metals, notably aluminum, have proved that section size is not a factor in determining proportional limit, provided the grain size is not excessive.[1] However, these tests were made on specimens varying from 0.500 to 0.050 inches in diameter, and wrought dental gold wires, in which the diameter is as small as 0.030 inches, are often used. It is possible, therefore, that a similar relationship does not exist in such a case.

A survey of the technical literature indicated that no analysis had been made of the effect of section size on the mechanical properties of the dental alloys. Such data would be of interest since they might reveal the validity of applying the published values for one size wire while a different size was being used in practice.

Previous work[2, 3] compared chemical composition, microstructure, and such mechanical properties as Vickers' diamond pyramid hardness, tensile proportional limit in bending, ultimate tensile strength and degree of set in bending. In this report two additional factors were considered; the effect of cross section of the wires, and the influence of high temperatures during the softening process on these properties. This last test was limited to the wire least influenced by section size using the proportional limits in bending and in tension as a criterion. Tests were conducted in the softened (as quenched) as well as the hardened states in the three wires investigated.

MATERIALS

All alloys were the standard commercial products, representing the composition and property range normally met with in wrought gold dental alloys.* They were in the form available to the profession, as wrought wires with the heat treatment, if any, that is normally performed by the producer before release. The alloys were obtained as 20 gauge (0.030 inch), 18 gauge (0.040 inch), 16 gauge (0.050 inch), and 14 gauge (0.064 inch) wires, suitable for tensile and bend tests. The chemical compositions of the alloys are given in Table I.

HEAT TREATMENT

All alloys were heat treated before testing. The wires had apparently been heat treated by the manufacturers, and it is obvious that this previous heat

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*The three alloys investigated were supplied by the following companies:
J. M. Ney Company, Hartford, Conn., Spyco Smelting and Refining Company., Minneapolis, Minn., The S. S. White Dental Manufacturing Company., Philadelphia, Pa.

TABLE I

CHEMICAL COMPOSITION OF DENTAL GOLD WIRES

ALLOY	ANALYSIS (PER CENT BY WEIGHT)						
	AU	PT	PD	AG	CU	ZN	NI
A	61.5	2.5	3.0	14.5	18.5	--	--
B	63.0	7.0	5.0	10.5	12.0	0.5	2.0
C	55.0	17.0	7.5	8.0	11.0	1.5	--
	ANALYSIS (ATOMIC PER CENT)						
A	40.1	1.6	3.6	17.3	37.4	--	--
B	43.8	4.9	6.4	13.4	25.8	1.0	4.7
C	39.5	12.3	10.0	10.5	24.5	3.2	--

treatment could influence the results obtained, particularly where the manufacturer's heat treatment took place at a temperature greater than that used at this laboratory to insure a softened state. However, after considering these possibilities, it was believed the best procedure would be to minimize such variations as much as possible by heating all wires to 1292° F. (700° C.) for 10 minutes then quenching in water. The hardened state was obtained by reheating for 15 minutes to 680° F. (360° C.) in a salt bath then quenching in water. In the case of wire *C*, a further heat treatment was carried out. One series of wires was heated 1700° to 1800° F. (927° to 982° C.) for one hour, then water quenched. This initial set was used for a series of bend tests. A second set of wires was heated 1700° to 1900° F. (927° to 1038° C.) for one hour, then water quenched. The higher temperature in this case was due to lack of control on the furnace, a small laboratory type dental unit. Since the higher temperature was used solely as a qualitative measure in determining its effect on grain size and variation in mechanical properties among the different size wires, this fluctuation in temperature was not considered to be of importance.

TABLE II

MECHANICAL PROPERTIES OF DENTAL GOLD WIRES

ALLOY	WIRE GAUGE	PROPORTIONAL LIMIT (TENSION)		PROPORTIONAL LIMIT (BENDING)	
		SOFT	HARD	SOFT	HARD
A	20	41,400	97,600	71,000	146,800
	18	50,900	100,600	82,100	156,200
	16	56,400	98,500	88,000	155,000
	14	55,200	101,800	88,800	162,500
B	20	47,500	107,600	96,000	146,300
	18	70,300	116,200	123,000	179,800
	16	87,900	123,100	127,300	194,700
	14	89,600	129,700	155,800	212,800
C	20	74,300	91,300	106,100	127,400
	18	73,900	93,100	119,400	148,300
	16	75,000	90,200	124,500	144,900
	14	73,500	93,900	121,200	153,900
CG	20	48,500		62,000	
	18	55,500		87,000	
	16	63,600		103,900	
	14	59,500		99,800	

TEST PROCEDURES

Tensile and bend tests as well as hardness measurements and polishing techniques were performed in the manner discussed in previous reports.[2, 3] A Tinius Olsen mechanical drive machine was used for the tensile tests. Bend tests were made on a Tinius Olsen stiffness tester using 2-inch wire lengths, and hardnesses were determined with a Vickers hardness tester using a 10 kg. load and a 136° diamond pyramid point.

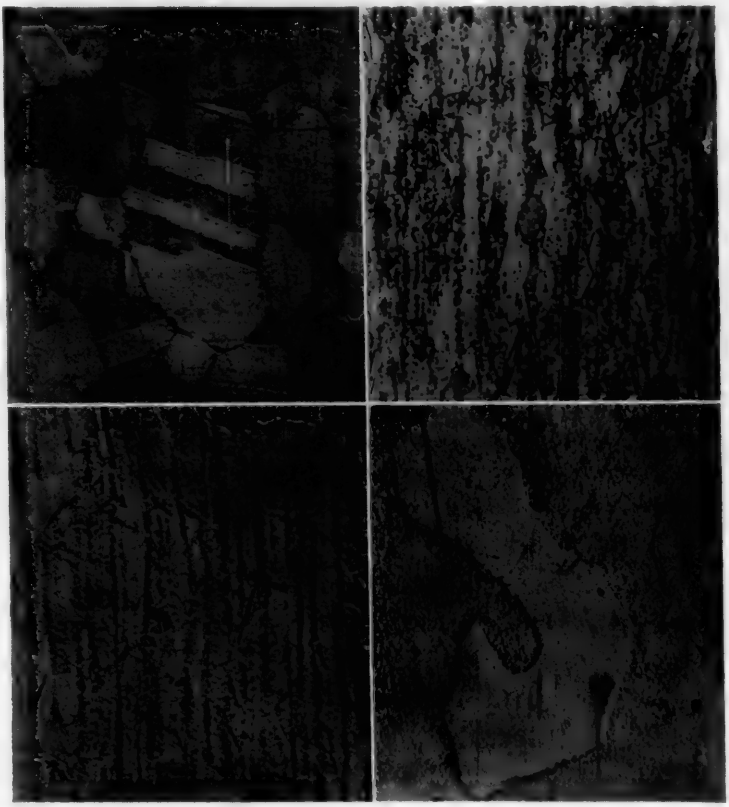

Fig. 1.—Microstructures of wrought gold wires. Potassium cyanide—Ammonium Persulfate Etch. 1000 diameters. *A*, Alloy *A*, soft. *B*, Alloy *B*, soft. *C*, Alloy *C*, soft. *D*, Alloy *CC*.

RESULTS

Proportional limit in bending and in tension was selected as a criterion for comparison, and these are the only values tabulated in Table II.

The microstructures of the three alloys are given at 1000 diameters in Fig. 1 in the soft (as quenched) state. An additional microstructure is also given in Fig. 1. This illustrates the effect on the grain size of alloy *C* of heating

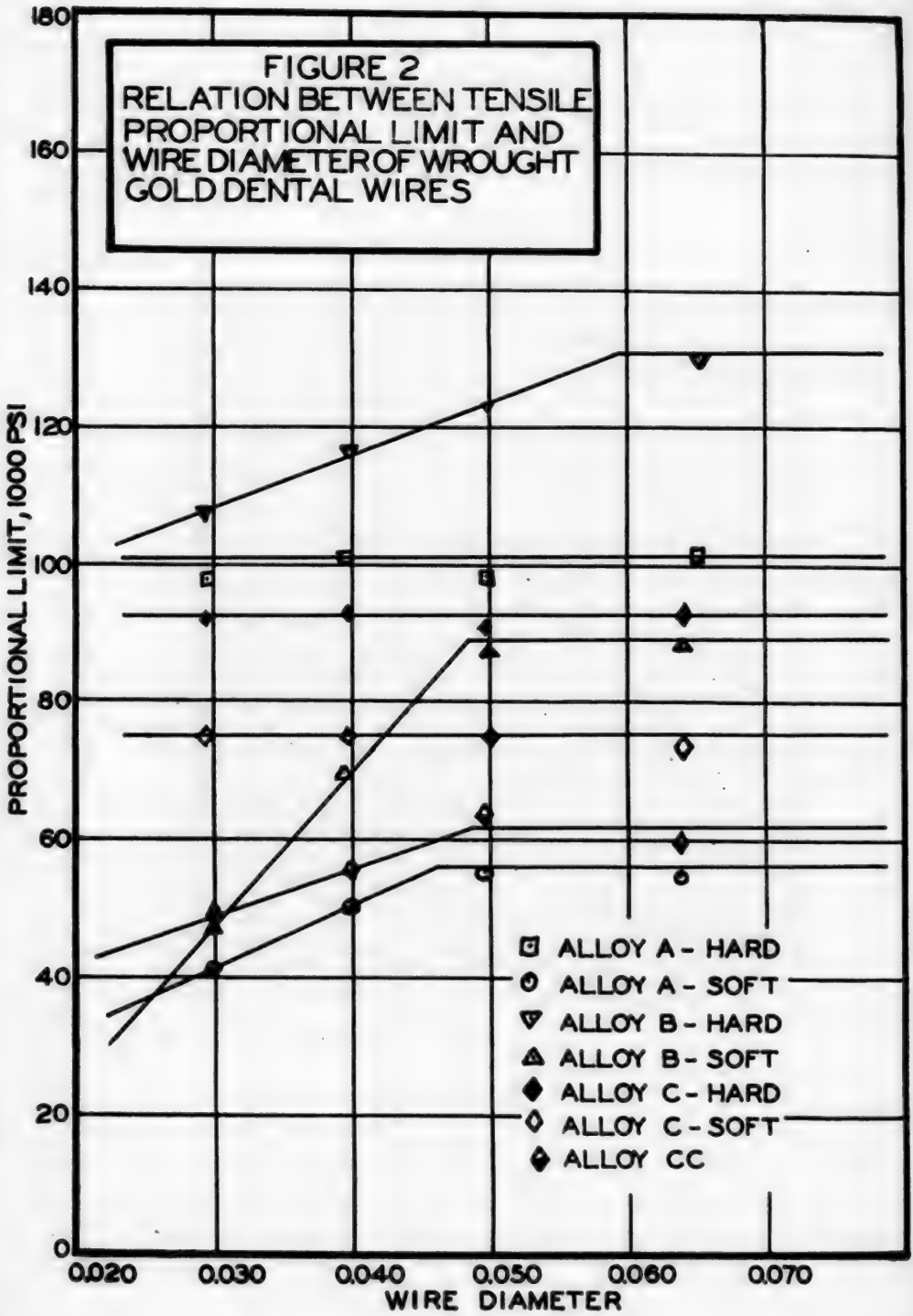
FIGURE 2
RELATION BETWEEN TENSILE PROPORTIONAL LIMIT AND WIRE DIAMETER OF WROUGHT GOLD DENTAL WIRES
PROPORTIONAL LIMIT, 1000 PSI
180
160
140
120
100
80
60
40
20
0
0.020
0.030
0.040
0.050
0.060
0.070
WIRE DIAMETER
ALLOY A - HARD
ALLOY A - SOFT
ALLOY B - HARD
ALLOY B - SOFT
ALLOY C - HARD
ALLOY C - SOFT
ALLOY CC

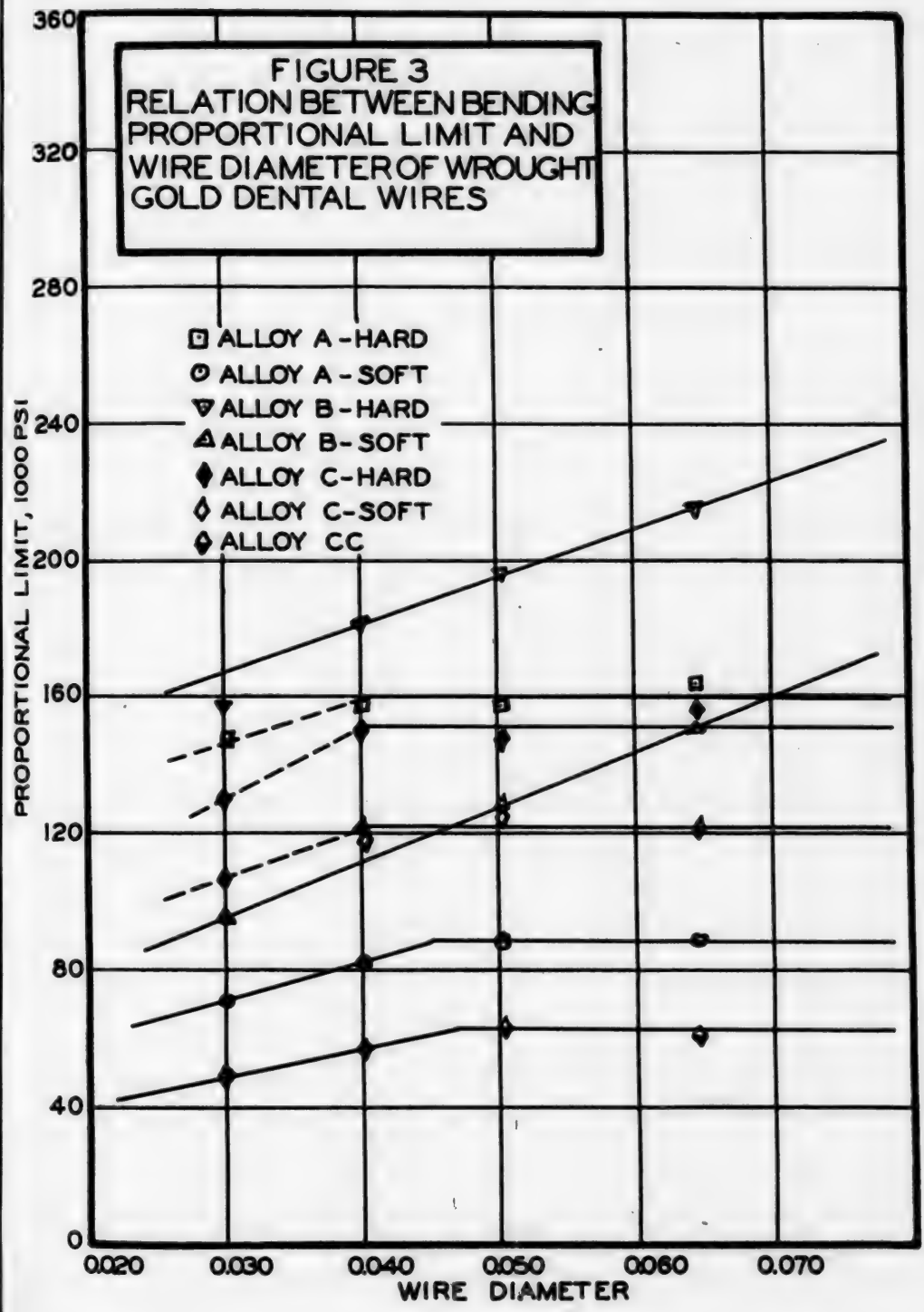
FIGURE 3
RELATION BETWEEN BENDING PROPORTIONAL LIMIT AND WIRE DIAMETER OF WROUGHT GOLD DENTAL WIRES
ALLOY A - HARD
ALLOY A - SOFT
ALLOY B - HARD
ALLOY B - SOFT
ALLOY C - HARD
ALLOY C - SOFT
ALLOY CC
PROPORTIONAL LIMIT, 1000 PSI
360
320
280
240
200
160
120
80
40
0
0.020
0.030
0.040
0.050
0.060
0.070
WIRE DIAMETER

to 1700° to 1800° F. for one hour. The photomicrographs aid in predicting the effect of grain size on the mechanical properties of wires as a function of the section size.

The difference in grain size from alloy to alloy, as well as that occurring in one particular alloy due to a variation in the heat treatment, is sufficient to infer qualitatively the existence of a critical section size below which the proportional limits decrease with a decrease in diameter. Above this critical cross section no change in mechanical properties should be noted with an increasing diameter.

DISCUSSION

In Figs. 2 and 3 are plotted the proportional limit in tension and proportional limit in bending versus the wire diameter, definitely indicating that no correlation exists between these mechanical properties and the section size of the wire. Examination of Fig. 2, proportional limit in tension versus wire diameter, does indicate that the different alloys, after the manufacturer's heat treatment and the softening heat treatment, did not respond in the same fashion. Alloy *A* has a critical diameter of about 0.044 inch, below which the proportional limit decreases with a decrease in diameter, and above which no change occurs with an increase in diameter. Alloy *B* responds in a similar fashion. In this case the critical diameter occurs at 0.048 inch. Alloy *C*, however, has a critical diameter less than that of the smallest wire tested (0.030 inch) since there is no change in proportional limit noted from the largest to the smallest wire. To determine the effect of excessively high heat treatment temperatures on an alloy, the least responsive, *C*, was heated within a temperature range varying from 1700° to 1900° F. and held there for one hour before being quenched in water. These tests, designated by the code letters *CC*, had lower proportional limits, a higher critical diameter (0.048 inch), and an increased grain size.

An examination of the proportional limits in tension as a function of the diameter indicates that the wires are generally less structure-sensitive in the hardened than in the softened state. Alloy *A* apparently has a critical section size slightly greater than or, possibly, less than 0.030 inch. Alloy *C* responds in the same fashion. The response of Alloy *B* differs from that of the other two. The results indicate that the critical section size is definitely increased. It is not possible to tell whether it is greater than 0.064 inch, but it cannot be much less in any event. No adequate explanation of this phenomenon is advanced due to lack of sufficient data; however, it is suggested that the precipitation hardening which is so pronounced in this particular alloy may be partially responsible.

The results of proportional limit in bending versus wire diameter are shown in Fig. 3. Analysis of the alloys in the softened state indicates that the results are comparable to those obtained in the tensile tests. In Alloy *A* the critical diameter is 0.045 inch. Apparently Alloy *B* is more structure-sensitive in bending than in tension, since the critical diameter is greater than 0.064 inch. Alloy *C* is also slightly more structure-sensitive. In tension the critical diameter

was less than 0.030 inch. In bending the critical diameter is approximately 0.040 inch. Alloy *CC* has a greater critical diameter than Alloy *C*, as would be expected. Here, the critical diameter is 0.046 inch.

The hardened wires again responded in bending in a fashion similar to that observed in the tensile tests. Alloy *A* had a critical diameter between 0.030 inch and 0.040 inch, somewhat higher than was observed in the tensile results. Alloy *C* also fell in this same range, again higher than in the tensile tests. In the case of Alloy *B* the critical diameter was greater than 0.064 inch, duplicating the results of the tensile tests.

It is apparent that either tensile or bend tests are satisfactory for determining the critical section size. In some cases the slightly greater sensitivity noted at smaller diameters, when using the bend test, indicates it might be preferable, particularly since the test condition is more comparable to conditions in practice than is a tensile test.

A comparison of the photomicrographs in Fig. 1 indicates that grain size is not the sole factor in determining the critical diameter. This is understandable when the wide variation in chemical composition is noted together with the definite over-all difference in shape of grain and type of precipitate occurring in the various alloys. In Alloy *C*, where such variables as chemical composition and type of matrix precipitate are eliminated, the increase in grain size accompanying the heat treatment at an excessive temperature can be considered to be one of the most important factors in shifting the critical diameter upward. It probably is not the sole factor, but it undoubtedly is responsible for some of the decrease in the proportional limit and the shift in critical section size.

CONCLUSIONS

1. No definite correlation exists between cross-section of the wire in either the soft or hard state and proportional limit in bending or in tension.

2. A critical section size exists below which the mechanical properties change as the section size decreases, and above which, no change in mechanical properties is noted with an increase in section size.

3. An increase in the platinum content is accompanied by a decrease in the critical section size. This is attributed to the refinement in grain size which accompanies such an increase in platinum.

4. An alloy with a small grain size is less structure-sensitive than an alloy with a large grain size. In the same alloy an increase in grain size is accompanied by a similar increase in the critical section size.

5. If the wires are heat-treated at an excessively high temperature, an increase in the grain size, and a decrease in the proportional limits in bending and in tension, will occur together with an increase in the critical section size, provided, of course, that the wires have not been subjected to a previous heat treatment at a higher temperature.

6. Both tensile and bend tests are satisfactory for determining the critical section size in hard or soft wires; however, the results indicate that the bend test may have a greater degree of section sensitivity than the tensile tests, at least in certain alloys.

7. Published values of the proportional limits of various alloys, when based on a single wire diameter, should be used for design purposes only when the design structure is equal to or greater in cross-section than the cross-section of the test wire. This precaution is unnecessary if it is known that the critical cross-section is smaller than either the design or test wire cross-section.

8. Alloys in which a pronounced precipitation hardening occurs are apparently much more structure sensitive in the hardened than in the softened state. On hardening, the critical section size is shifted to a much greater value in both tensile and bend tests.

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Errata

In Abstract 18 (*J. D. Res.* **30**: 465, 1951), line 7 on page 466 should read "The precarious lesions," not "The carious lesions."

In Abstract 121 (*J. D. Res.* **30**: 504, 1951), line 2 on page 505 should read "erupted tooth," and not "unerupted tooth."

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